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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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 ($\Delta\Psi$) 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 $\Delta\Psi$, 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 $\Delta\Psi$ 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 $600 \times g$ for 5 min. The supernatant was then centrifuged at $9,000 \times g$ 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$_2$PO$_4$, 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$_2$O$_2$) 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.
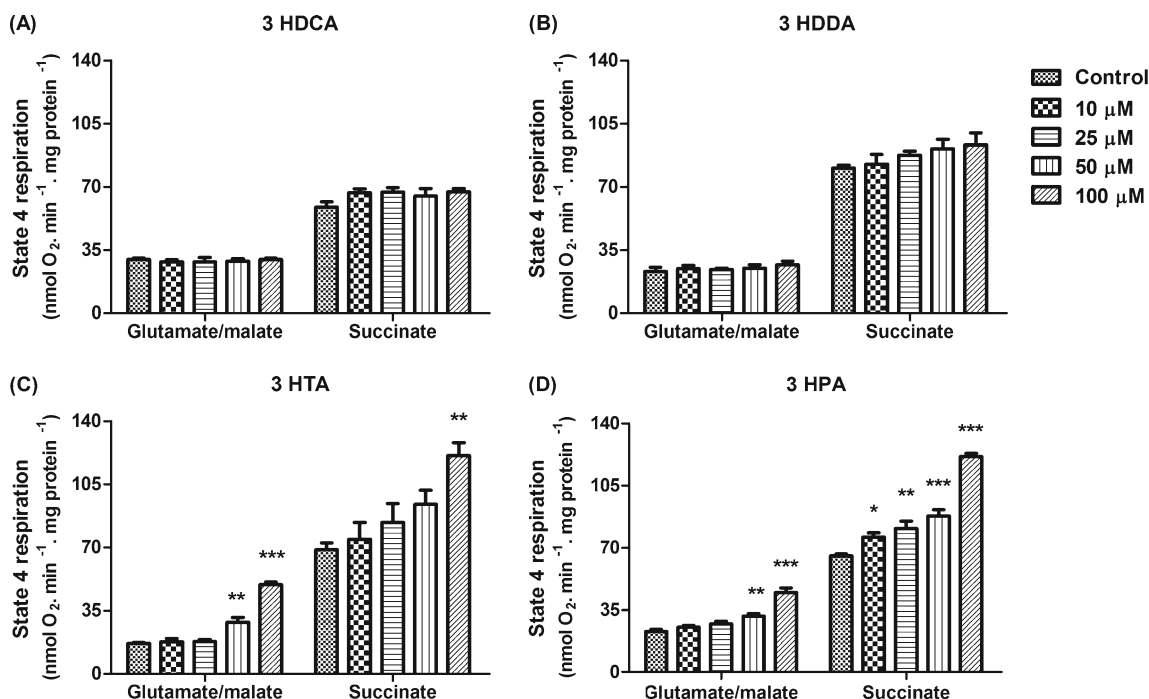
Fig. 1 Effect of 3-hydroxydecanoic (3 HDCA) (a), 3-hydroxydodecanoic (3 HDDA) (b), 3-hydroxytetradecanoic (3 HTA) (c) and 3-hydroxypalmitic (3 HPA) (d) acids on state 4 respiration using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (0.2 mg protein. 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)
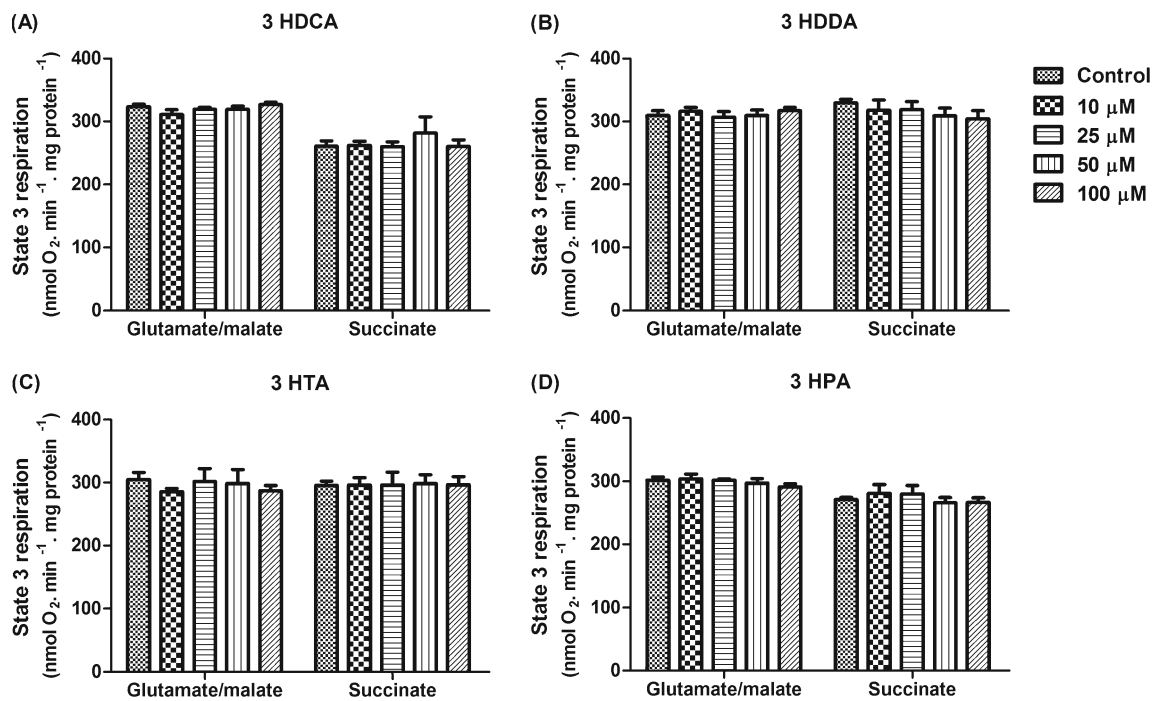
Fig. 2 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on state 3 respiration using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial preparations (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$_2$O$_2$) production in the presence of succinate as substrate. We found that 3 HDDA, 3 HTA and 3 HPA significantly reduced H$_2$O$_2$ production (Fig. 6), which is also caused by uncouplers. The inhibitory effect of LC3HFA on mitochondrial H$_2$O$_2$ 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+}
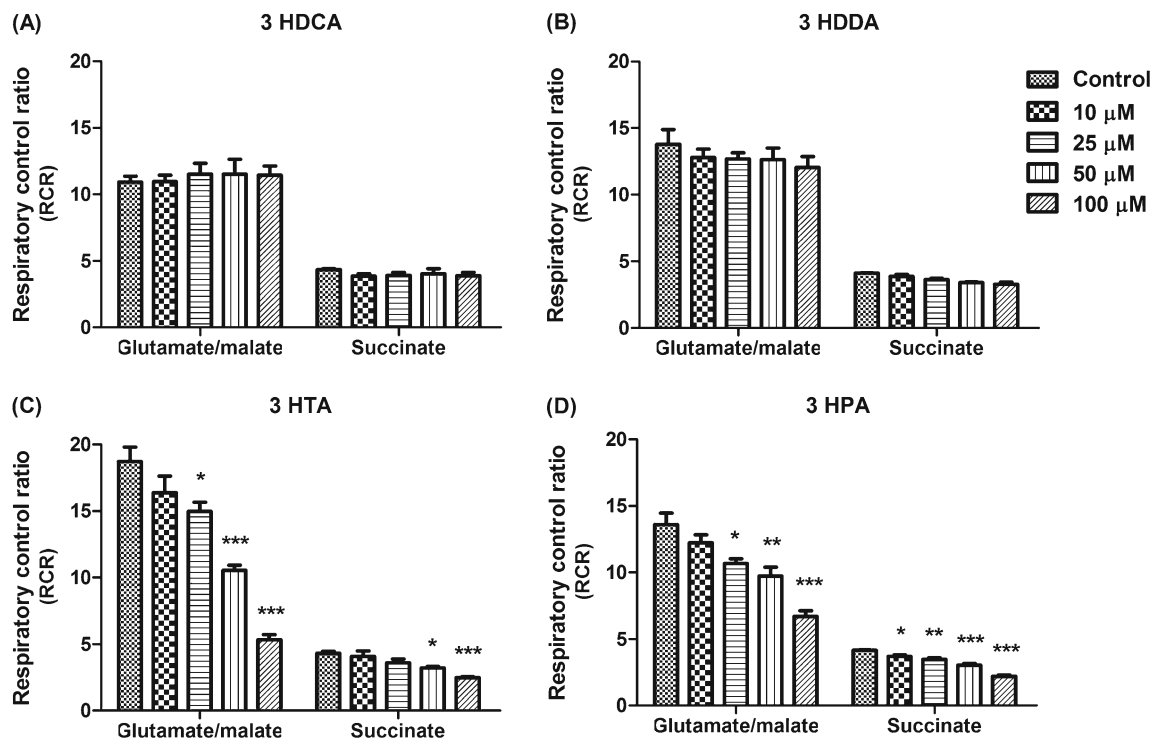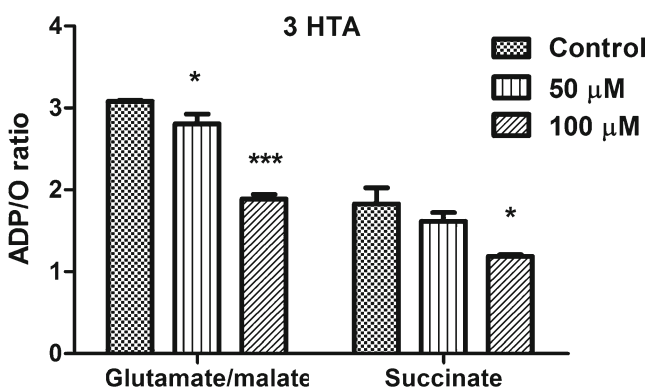
Fig. 3 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on respiratory control ratio (RCR) using glutamate/malate or succinate plus rotenone (4 μM) as substrates. Heart mitochondrial 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

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)

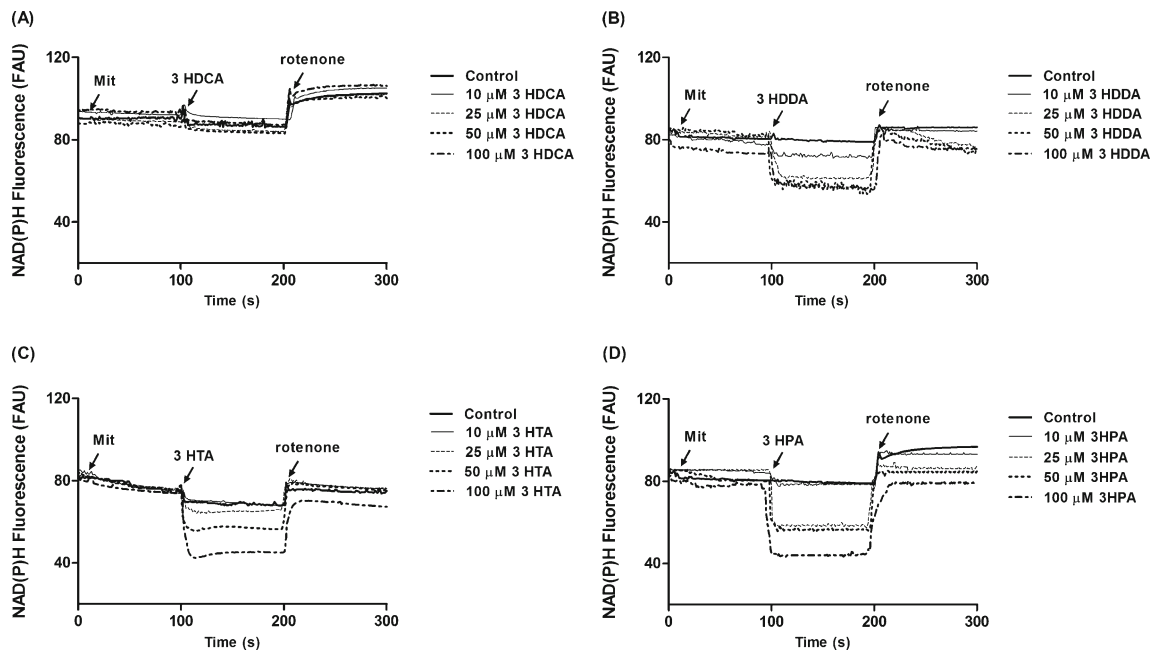effects on ΔΨ in heart mitochondrial preparations supplemented by Ca^{2+}.

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;
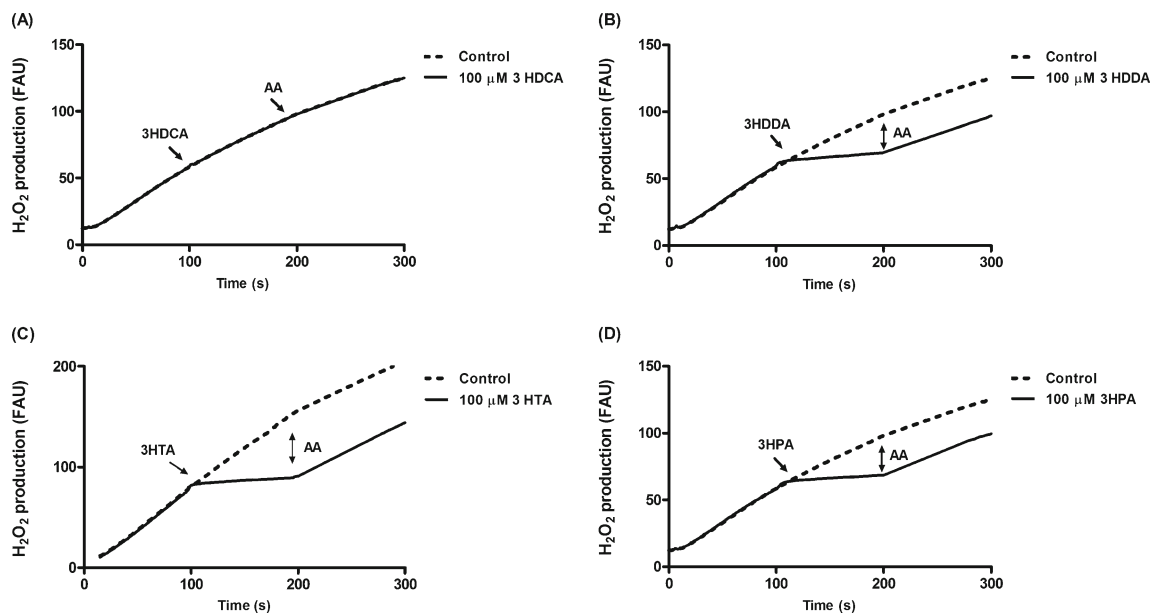
Fig. 5 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on NAD(P)H content using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.2 mg protein. mL^{-1}) and 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)
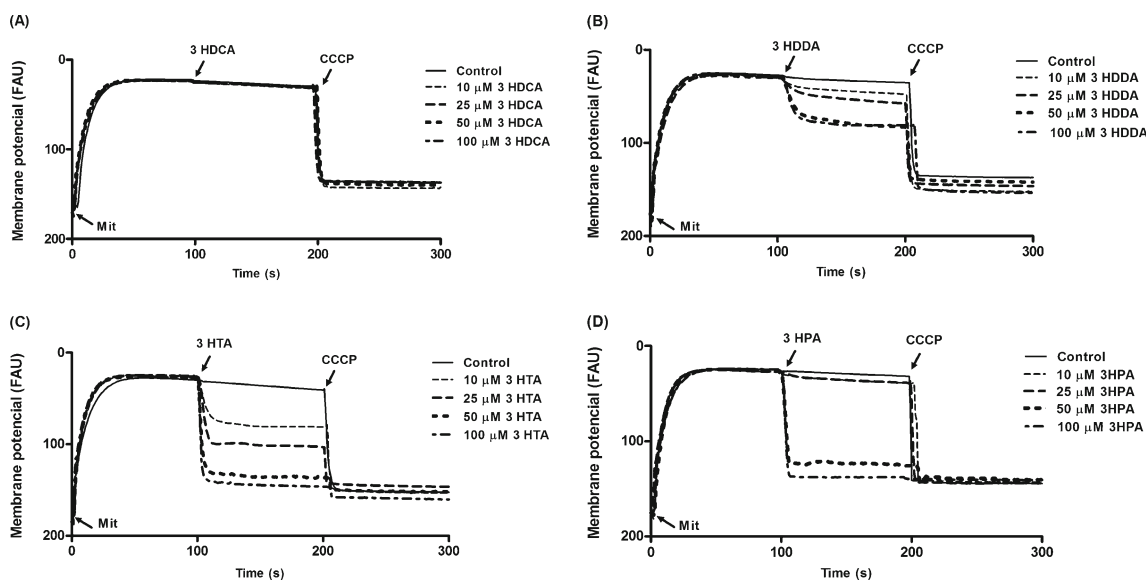
Fig. 7 Effects of 3-hydroxydecanoic (3 HDCA) (**a**), 3-hydroxydodecanoic (3 HDDA) (**b**), 3-hydroxytetradecanoic (3 HTA) (**c**) and 3-hydroxypalmitic (3 HPA) (**d**) acids on mitochondrial membrane potential ($\Delta\Psi$) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit., 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
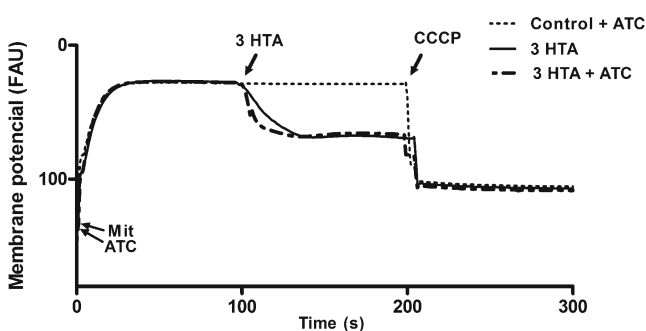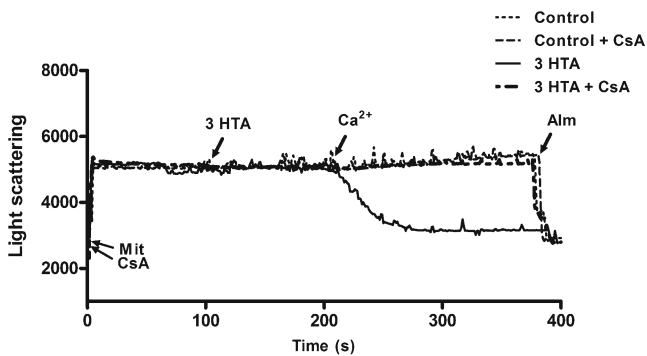

Fig. 8 Effect of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial membrane potential ($\Delta\Psi$) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.3 mg protein. mL^{-1}) were added to the incubation medium, as indicated by the arrow, in the presence of 5 μM safranine 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)
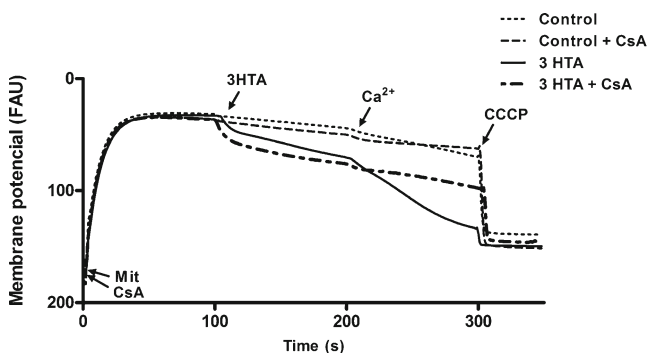
Fig. 9 Effect of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial membrane potential ($\Delta\Psi$) using glutamate/malate as substrates. Heart mitochondrial preparations (Mit.; 0.3 mg protein. mL^{-1}) were added to the incubation medium, as indicated by the arrow, in the presence of 5 μM safranine 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 trans-bilayer 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$_2$O$_2$ 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.

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

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