Uncoupling, metabolic inhibition and induction of mitochondrial permeability transition in rat liver mitochondria caused by the major long-chain hydroxyl monocarboxylic fatty acids accumulating in LCHAD deficiency

CrossMark

Fernanda Hermes Hickmann [a], Cristiane Cecatto [a], Daniele Kleemann [a], Wagner Oliveira Monteiro [a], Roger Frigério Castilho [c], Alexandre Umpierrez Amaral [a], Moacir Wajner [a,b,*]

[a] Departamento de Bioquímica, Instituto de Ciências Básicas da Saúde, Universidade Federal do Rio Grande do Sul, Porto Alegre, RS, Brazil
[b] Serviço de Genética Médica, Hospital de Clínicas de Porto Alegre, Porto Alegre, RS, Brazil
[c] Departamento de Patologia Clínica, Faculdade de Ciências Médicas, Universidade Estadual de Campinas, Campinas, SP, Brazil

ARTICLE INFO

ABSTRACT

Patients with long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) deficiency commonly present liver dysfunction whose pathogenesis is unknown. We studied the effects of long-chain 3-hydroxylated fatty acids (LCHFA) that accumulate in LCHAD deficiency on liver bioenergetics using mitochondrial preparations from young rats. We provide strong evidence that 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids, the monocarboxylic acids that are found at the highest tissue concentrations in this disorder, act as metabolic inhibitors and uncouplers of oxidative phosphorylation. These conclusions are based on the findings that these fatty acids decreased ADP-stimulated (state 3) and uncoupled respiration, mitochondrial membrane potential and NAD(P)H content, and, in contrast, increased resting (state 4) respiration. We also verified that 3HTA and 3HPA markedly reduced Ca^{2+} retention capacity and induced swelling in Ca^{2+}-loaded mitochondria. These effects were mediated by mitochondrial permeability transition (MPT) induction since they were totally prevented by the classical MPT inhibitors cyclosporin A and ADP, as well as by ruthenium red, a Ca^{2+} uptake blocker. Taken together, our data demonstrate that the major monocarboxylic LCHFA accumulating in LCHAD deficiency disrupt energy mitochondrial homeostasis in the liver. It is proposed that this pathomechanism may explain at least in part the hepatic alterations characteristic of the affected patients.

© 2015 Elsevier B.V. All rights reserved.

1. Introduction

The mitochondrial enzyme long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD, EC 1.1.1.211) is responsible for the third step in the oxidation of long-chain fatty acids (LCFA). Deficiency of LCHAD was described in 1989 [1] and is a relatively frequent fatty acid oxidation defect [2]. It is biochemically characterized by accumulation of long-chain 3-hydroxylated fatty acids (LCHFA) in body fluids and tissues, as well as by episodes of lactic acidemia and hypoketotic hypoglycemia, particularly during periods of intense catabolism, such as prolonged fasting and infections [3–6]. The clinical manifestations of LCHAD deficiency are more severe than the β-oxidation disorders of medium and short chain fatty acids [7,8], suggesting a high toxicity for the accumulating LCHFA. Affected patients present a wide variety of symptoms, including severe hepatopathy and cardiomyopathy, as well as retinopathy, hypotonia, peripheral neuropathy, speech and developmental delay, lethargy and seizures [9,10].

Hepatic dysfunction is a common finding in LCHAD deficiency, especially during episodes of metabolic decompensation, which are characterized by hypoketosis hypoglycemia, hyperlactic acidemia and lethargy [11]. Most patients present hepatomegaly, acute cholestatic

jaundice, massive hepatic necrosis and hepatic steatosis accompanied by fibrosis [12–14].

Treatment is based on dietary fat restriction, fasting avoidance, night feeds, as well as supplementation of essential fatty acids and medium chain triglycerides [15–18].

The pathophysiology of tissue damage in LCHAD deficiency is still unclear, but may possibly be associated with hypoglycemia and hypoketonemia leading to energy deprivation. Therefore, since liver essentially utilizes fatty acids for most of its energetic needs, the hepatic symptomatology of LCHAD deficiency may involve low energy production from fatty acids and/or alternatively due to the toxicity of LCHFA or their long-chain 3-hydroxyacylcarnitines derivatives that accumulate in this disorder [19–22]. Noteworthy, hyperlactic acidemia associated with

abnormal mitochondrial morphology with swollen appearance and fat infiltration observed in these patients indicates a role for mitochondrial energy disruption in its ethiopathogenesis [23]. Recent experimental studies demonstrating that the monocarboxylic LCHFA accumulating in LCHAD deficiency provoke impairment of mitochondrial bioenergetics in forebrain and heart of young rats, strengthen this hypothesis [24,25]. In a further report, it was demonstrated that LCHFA induce mitochondrial permeability transition (MPT) in Ca^{2+}-loaded brain mitochondria [26]. These data provide unequivocal evidence that LCHFA disturb energy mitochondrial homeostasis in brain and heart. However, to the best of our knowledge, there is no work evaluating mitochondrial function in liver exposed to the LCHFA accumulating in LCHAD deficiency.
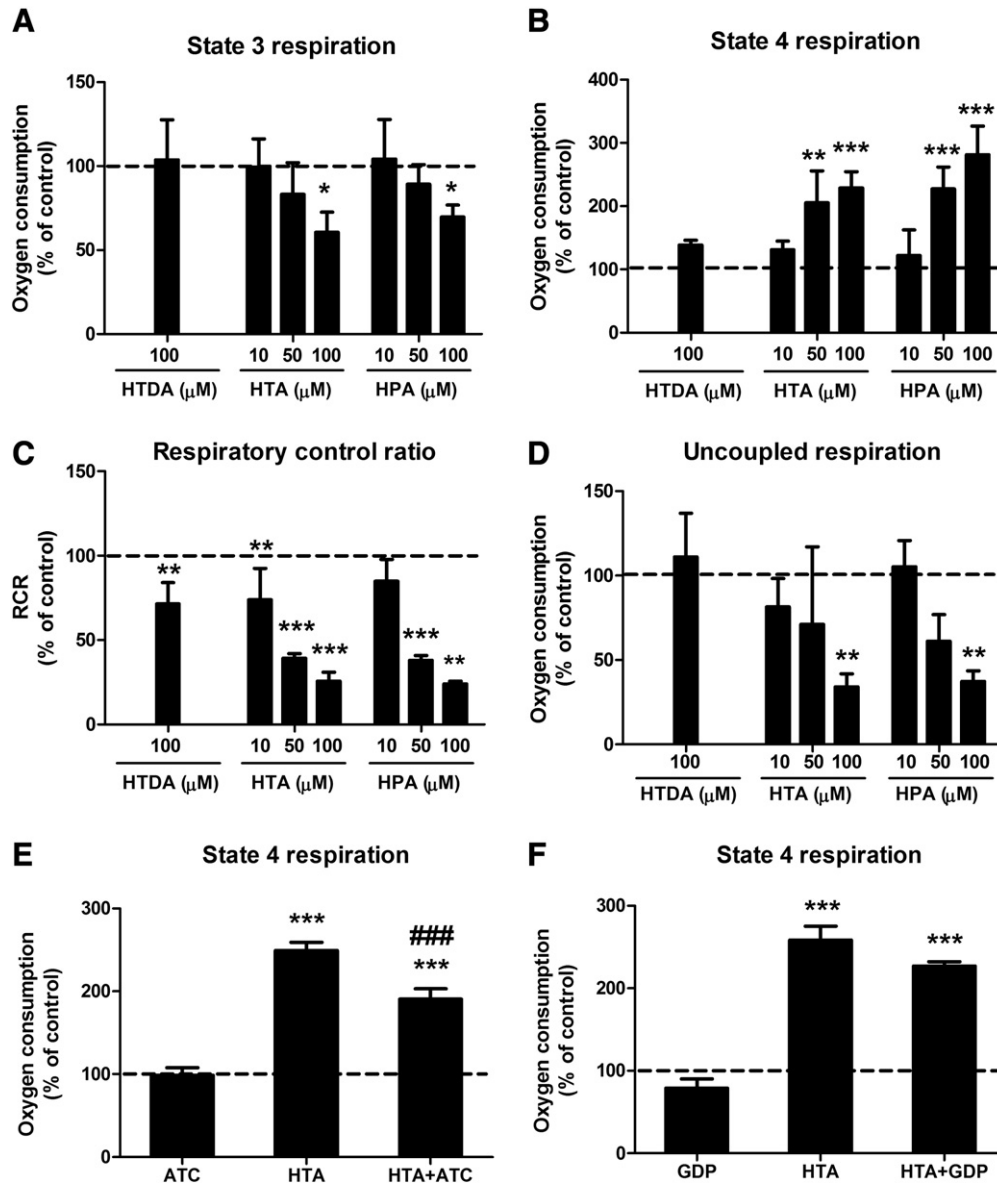

Fig. 1. Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on ADP-stimulated (state 3) (A), resting (state 4) (B), and uncoupled (CCCP-stimulated) (D) respiration, as well as respiratory control ratio (RCR) (C), using glutamate/malate as substrate in liver mitochondria. Panels E and F show the effects of 3HTA (100 μM) on resting (state 4) respiration in the presence of atractyloside (ATC, 30 μM) and guanosine diphosphate (GDP, 200 μM), respectively. The mitochondrial preparations (0.75 mg protein·mL^{-1}) and 3HTDA, 3HTA or 3HPA (10–100 μM) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids and ethanol (the final concentration of EtOH in the incubation medium was 1%) was added to the incubation medium in the beginning of the assays. The reaction media contained 200 μM EGTA. Values are means ± standard deviation for three to six independent experiments and are expressed as percentage of control (Controls: (A) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 69.02 ± 11.94; (B) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 14.62 ± 3.58; (C) [RCR] 4.95 ± 1.12; (D) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 53.40 ± 13.18; (E) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 13.51 ± 2.66; (F) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 11.92 ± 2.56). *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls. ###P < 0.001, compared to HTA (Duncan's multiple range test).

Thus, in the present work we investigated the role of the 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on important parameters of mitochondrial bioenergetics, namely the respiratory states 3 and 4, respiratory control ratio (RCR) and uncoupled respiration, as well as the mitochondrial membrane potential ($\Delta\Psi m$), swelling, Ca^{2+} retention capacity and NAD(P)H content in liver mitochondrial preparations from young rats in the presence or absence of Ca^{2+} in the hopes to clarify the mechanisms of toxicity of these compounds.

2. Material and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA), except for 3HTDA (96% purity), which was synthesized by Dr. Ernesto Brunet, from Universidad Autonoma de Madrid. Stock solutions of a racemic mixture (DL) of 3HTDA, 3HTA and 3HPA were prepared in ethanol (EtOH) to dissolve the fatty acids and added to incubation medium at final concentrations of 10–100 μM. The final concentration of EtOH in the incubation medium was 1%. The same percentage of EtOH was present in controls and proved not to alter *per se* the parameters evaluated.

2.2. Animals

Thirty-day-old Wistar rats obtained from our breeding colony were used. The animals were maintained on a 12:12 h light/dark cycle in air-conditioned constant temperature (22 ± 1 °C) colony room, with free access to water and 20% (w/w) protein commercial chow. This study was performed in strict accordance with the Guide for the Care and Use of Laboratory Animals (eighth edition, 2011) and approved by the Ethical Committee of the Federal University of Rio Grande do Sul, Porto Alegre, Brazil. All efforts were also made to use the minimal number of animals necessary to produce reliable scientific data. The manuscript does not contain clinical studies or patient data.

2.3. Preparation of mitochondrial fractions

Mitochondrial preparations from liver were isolated according to Mirandola [27], with modifications. Animals were sacrificed by decapitation, and the liver was removed and homogenized with a glass handheld homogenizer in ice-cold mitochondria isolation medium 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 2000 ×g for 3 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12,000 ×g for 10 min at 4 °C. The resultant pellet was resuspended in 5 mL of isolation medium without EGTA and centrifuged at 12,000 ×g for 10 min at 4 °C. The final pellet was resuspended in isolation medium without EGTA in an approximate protein concentration of 30 mg·mL^{-1}. Protein concentration was measured by the method of Bradford [28], using BSA as standard. Mitochondria obtained from liver were used in the assays immediately after isolation and assays were carried out in the absence or presence of Ca^{2+}. In some experiments, we used mitochondria from forebrain whose preparation was similar to the liver [27].
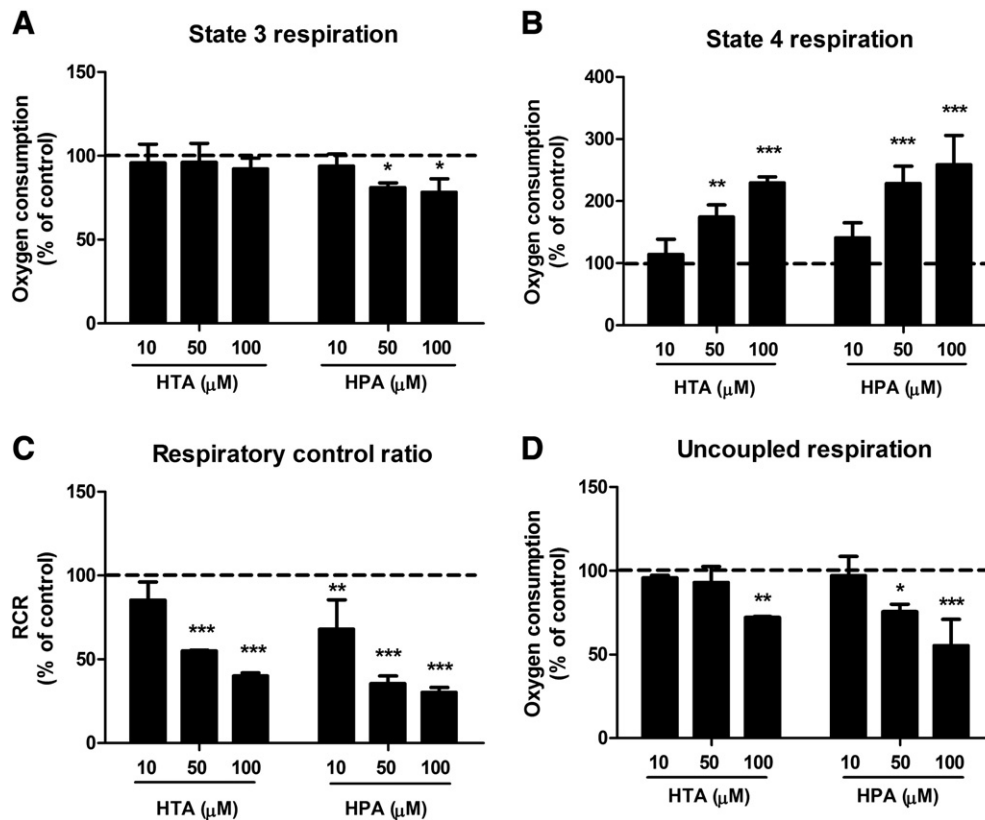

Fig. 2. Effects of 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on ADP-stimulated (state 3) (A), resting (state 4) (B) and uncoupled (CCCP-stimulated) (D) respiration, as well as respiratory control ratio (RCR) (C), using succinate as substrate plus rotenone in liver mitochondria. The mitochondrial preparations (0.5 mg protein·mL^{-1}) and various concentrations of 3HTA or 3HPA (10–100 μM) were added to the incubation medium in the beginning of the assays. Controls were performed in the absence of fatty acids and ethanol (the final concentration of EtOH in the incubation medium was 1%) was added to the incubation medium in the beginning of the assays. The reaction media contained 200 μM EGTA. Values are means ± standard deviation for three independent experiments and are expressed as percentage of control (Controls: (A) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 125.60 ± 8.21; (B) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 34.55 ± 3.91; (C) [RCR] 3.66 ± 0.43; (D) [nmol O$_2$·min^{-1}·mg of protein^{-1}]: 116.75 ± 17.95). *P < 0.05, **P < 0.01, ***P < 0.001, compared to controls (Duncan's multiple range test).

2.4. 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. 3HTDA, 3HTA or 3HPA (10–100 μM) was added to the reaction medium at the beginning of the assay. The assay was performed with mitochondrial preparations (0.75 mg protein^{-1}·mL^{-1} when using 2.5 mM glutamate plus 2.5 mM malate as substrates and 0.5 mg protein^{-1}·mL^{-1} when using 5 mM succinate as substrate plus 4 μM rotenone) and incubated for 60 s in a buffer containing 100 mM sucrose, 65 mM potassium chloride, 10 mM HEPES, 1 mM KH$_2$PO$_4$ and 200 μM EGTA, pH 7.2. State 3 respiration was measured after addition of 1 mM ADP to the incubation medium and uncoupled respiration after addition of 3 μM CCCP. To measure

resting (state 4) respiration, 1 μg·mL^{-1} oligomycin A was added to the incubation medium. In some experiments, guanosine diphosphate (GDP, 200 μM) and atractyloside (ATC, 30 μM) were added to the mitochondrial preparations. The RCR (state 3/state 4) was then calculated. States 3 and 4 were expressed as nmol O$_2$ consumed·min^{-1}·mg protein^{-1}. Only mitochondrial preparations with RCR greater than 3 were used in the experiments.

2.5. Experimental procedures for the spectrofluorimetric and spectrophotometric assays

Measurements of $\Delta\Psi$m, NAD(P)H content and Ca^{2+} retention capacity were performed using spectrofluorimetry, whereas mitochondrial swelling was measured by spectrophotometry. Mitochondrial incubations were carried out at 37 °C, with continuous magnetic stirring
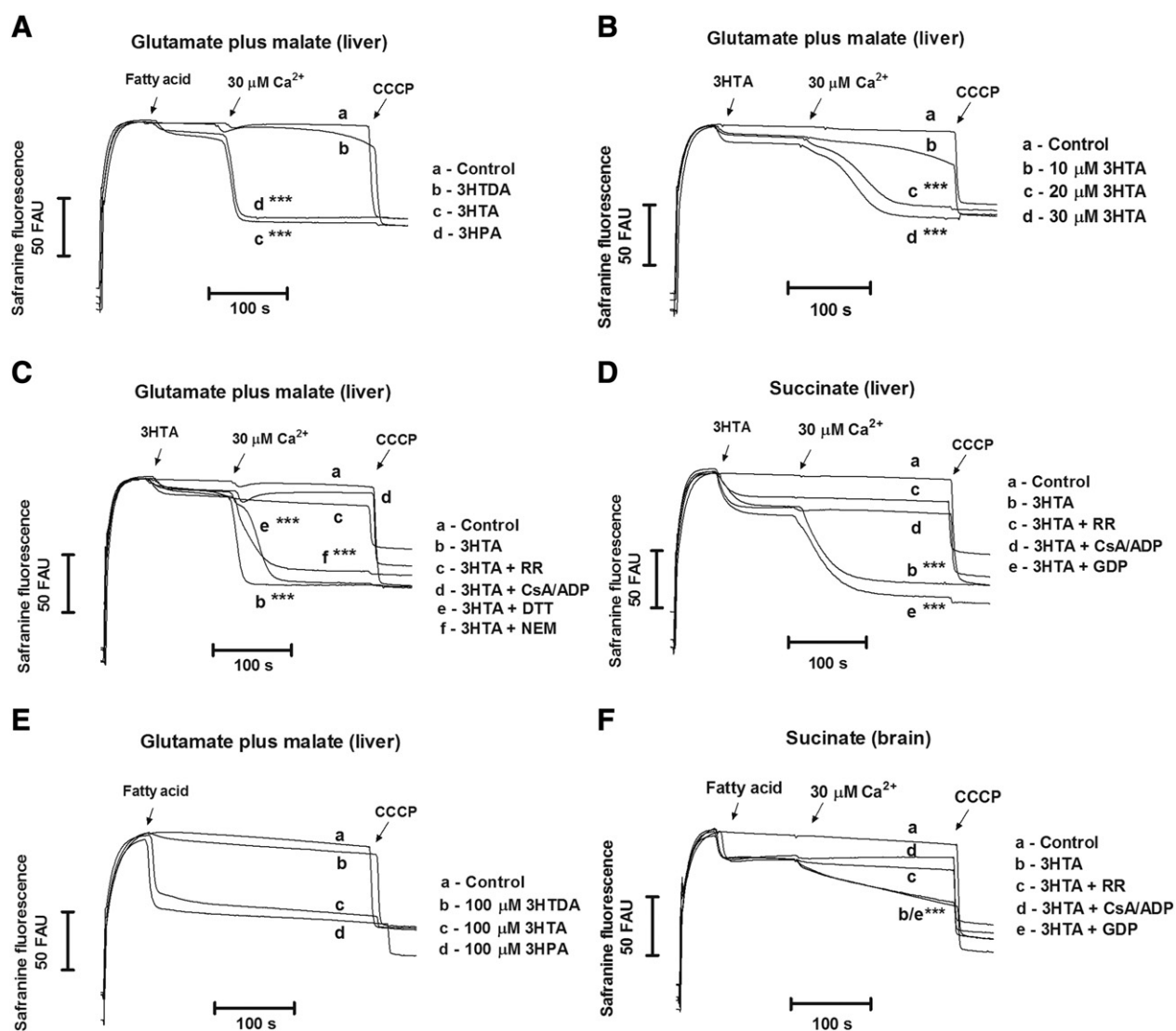

Fig. 3. Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial membrane potential in the presence (A–D and F) or absence (E) of Ca^{2+} in liver (A–E) and brain (F) mitochondria. All experiments were performed in a reaction media containing mitochondrial preparations (0.5 mg protein·mL^{-1}) supported by glutamate/malate or succinate. (A) 3HTDA, 3HTA or 3HPA (30 μM, lines b–d) were added 50 s after the beginning of the assay. (B) 3HTA (10–30 μM, lines b–d). (C) 3HTA (30 μM, lines b–f), ruthenium red (RR, 5 μM, line c), cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line d), dithiothreitol (DTT, 5 mM) (line e) or *N*-ethylmaleimide (NEM, 20 μM) (line f) was added in the beginning of the assay. (D) 3HTA (30 μM, lines b–e), ruthenium red (RR, 1 μM, line c), cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line d) and guanosine diphosphate (GDP, 200 μM, line e) were added in the beginning of the assay. (E) 3HTDA (100 μM, lines b), 3HTA (100 μM, lines c) and 3HPA (100 μM, lines d). (F) 3HTA (30 μM, lines b–e), ruthenium red (RR, 1 μM, line c), cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line d) and guanosine diphosphate (GDP, 200 μM, line e) were added in the beginning of the assay. Controls (lines a) were performed in the absence of fatty acids. Panels A–D and F refer to mitochondrial preparations supplemented by 30 μM Ca^{2+} 150 s after the beginning of the assay, as indicated, whereas in panel E there was no exogenous Ca^{2+}. The reaction media contained 30 μM (A–D and F) or 100 μM (E) EGTA. CCCP (3 μM) was added in the end of the assays. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU). ***P < 0.001, compared to controls (Duncan's multiple range test).

and the assays were conducted in the presence of 1 µg·mL^{-1} oligomycin A (resting respiration) using mitochondria (0.5 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate or 5 mM succinate plus 4 µM rotenone. 3HTDA (30–100 µM), 3HTA (10–100 µM), 3HPA (10–100 µM), CaCl$_2$ (20–40 µM), CCCP (3 µM) and alamethicin (Alm, 40 µg·mL^{-1}) were added as indicated by the arrows in the figures. In some experiments, ruthenium red (RR, 5 µM), cyclosporin A (CsA, 1 µM), ADP (300 µM), dithiothreitol (DTT, 5 mM), N-ethylmaleimide (20 µM, NEM) and GDP (200 µM) were added in the assay. Traces are representative of independent experiments carried out in mitochondrial preparations from liver of three animals and were expressed as arbitrary units, unless otherwise stated. Statistical analyses were also carried out by analyzing quantitatively the data obtained from the assays.

2.6. Mitochondrial membrane potential (ΔΨm)

The ΔΨm was estimated according to Akerman and Wikstrom [29] and Figueira and collaborators [30] in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM HEPES, 2 mM KH$_2$PO$_4$, 30 or 100 µM EGTA, pH 7.2. The fluorescence of 5 µM cationic dye safranine O, a ΔΨm indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added in the end of measurements to abolish ΔΨm.

2.7. Mitochondrial NAD(P)H

Mitochondrial matrix NAD(P)H fluorescence was measured at 366 nm excitation and 450 nm emission wavelengths, in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM HEPES, 2 mM KH$_2$PO$_4$, 30 or 100 µM EGTA, pH 7.2. CCCP was added in the end of the measurements to induce maximal NAD(P)H oxidation.

2.8. Mitochondrial swelling

Mitochondrial swelling was determined in 96-well plates as the decrease in the turbidity of the mitochondrial suspension measured at 540 nm wavelength using a SpectraMax M5 microplate reader, in a medium (final volume: 250 µL) containing 125 mM sucrose, 65 mM KCl, 2 mM K$_2$HPO$_4$, 1 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 10 mM HEPES, 15 µM EGTA, pH 7.2. A decrease in the turbidity indicates an increase in mitochondrial swelling. Alamethicin (Alm) was added in the end of the experiment to provoke maximal swelling.

2.9. Mitochondrial Ca^{2+} retention capacity

Ca^{2+} retention capacity was determined in a medium containing 150 mM KCl, 5 mM MgCl$_2$, 0.1 mg·mL^{-1} BSA, 5 mM HEPES, 2 mM KH$_2$PO$_4$, 10 µM EGTA, pH 7.2, following the external free Ca^{2+} levels using 0.2 µM Calcium Green-5N (Molecular Probes, Invitrogen, Carlsbad, CA) at excitation and emission wavelengths of 506 and 532 nm, respectively [31]. In the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

2.10. Statistical analysis

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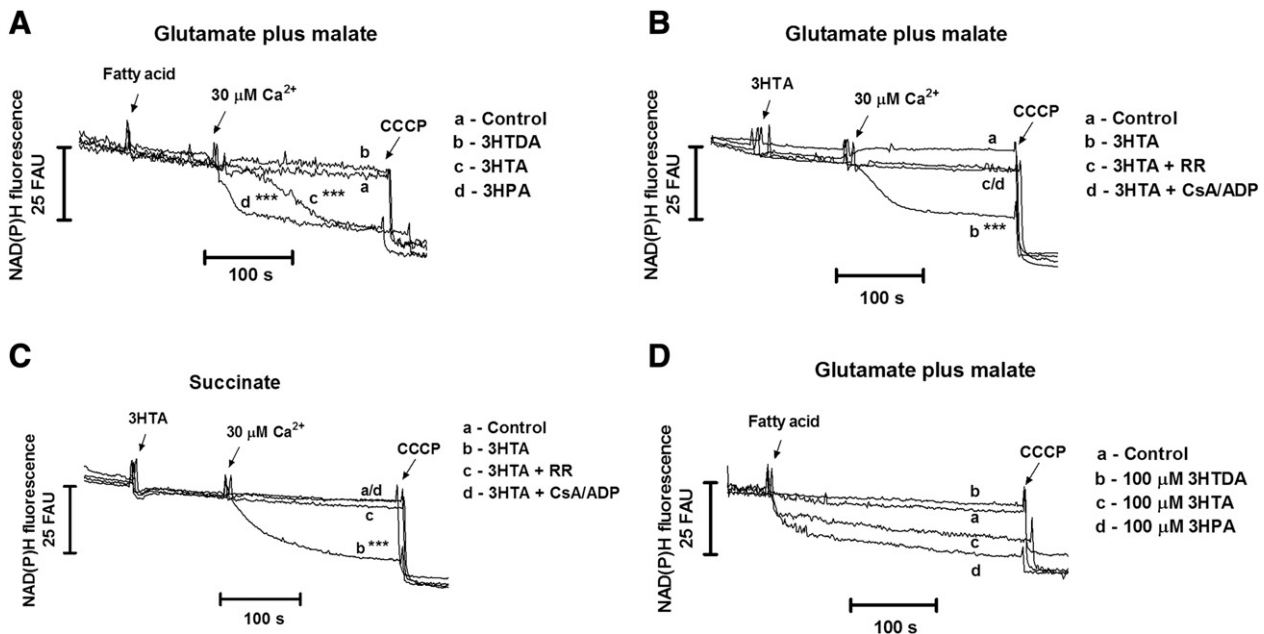

Fig. 4. Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial NAD(P)H content in the presence (A–C) or absence (D) of Ca^{2+}. All experiments were performed in a reaction media containing mitochondrial preparations (0.5 mg protein·mL^{-1}) supported by glutamate/malate or succinate. (A) 3HTDA, 3HTA or 3HPA (30 µM, lines b–d) was added 50 s after the beginning of the assay. (B) 3HTA (30 µM, lines b–d), ruthenium red (RR, 5 µM, line c) and cyclosporin A (CsA, 1 µM) plus ADP (300 µM) (line d) were added in the beginning of the assay. (C) 3HTA (30 µM, lines b–d). ruthenium red (RR, 1 µM, line c) and cyclosporin A (CsA, 1 µM) plus ADP (300 µM) (line d) were added in the beginning of the assay. (D) 3HTDA (100 µM, line b), 3HTA (100 µM, line c) and 3HPA (100 µM, line d). Controls (lines a) were performed in the absence of fatty acids. Panels A–C refer to mitochondrial preparations supplemented by 30 µM Ca^{2+} 150 s after the beginning of the assay, as indicated, whereas in panel D there was no exogenous Ca^{2+}. The reaction media contained 30 µM (A–C) or 100 µM (D) EGTA. CCCP (3 µM) was added in the end of the assays. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU). ***P < 0.001, compared to controls (Duncan's multiple range test).

3. Results

3.1. Long-chain monocarboxylic 3-hydroxylated fatty acids alter oxygen consumption in liver mitochondria

The effect of the 3HTDA, 3HTA and 3HPA on liver mitochondrial respiratory parameters measured by oxygen consumption using glutamate plus malate or succinate as respiratory substrates was first investigated. 3HTA and 3HPA (50 and 100 μM) markedly increased resting (state 4) (Fig. 1B: [$F_{(7,19)}$ = 12.92, P < 0.001]) and decreased ADP-stimulated (state 3) (Fig. 1A: [$F_{(7,19)}$ = 3.447, P < 0.05]), uncoupled respiration (Fig. 1D: [$F_{(7,18)}$ = 5.538, P < 0.01]) and RCR (Fig. 1C: [$F_{(7,19)}$ = 23.13, P < 0.001]) in a dose dependent manner with glutamate plus malate as substrates. Similar results were obtained with succinate-supported mitochondria (Fig. 2A: [$F_{(6,14)}$ = 3.054, P < 0.05]; Fig. 2B: [$F_{(6,14)}$ = 16.82, P < 0.001]; Fig. 2C: [$F_{(6,14)}$ = 24.73, P < 0.001]; Fig. 2D: [$F_{(6,14)}$ = 8.095, P < 0.01]). We also verified that the 3HTA-induced increase of resting respiration was attenuated by the competitive adenine nucleotide translocator (ANT) inhibitor ATC (Fig. 1E: [$F_{(3,20)}$ = 53.56, P < 0.001]), but not by the UCP inhibitor GDP (Fig. 1F: [$F_{(3,8)}$ = 53.19, P < 0.001]), suggesting a role for ANT in the increase of state 4 respiration provoked by 3HTA. In contrast, apart from a moderate reduction of RCR, overall 3HTDA was not able to significantly alter these respiratory parameters (Fig. 1).

3.2. Long-chain monocarboxylic 3-hydroxylated fatty acids reduce ΔΨm in liver and brain mitochondria

Next, we assessed the influence of 3HTDA, 3HTA and 3HPA on ΔΨm in liver mitochondria supported by glutamate plus malate or succinate in order to further investigate the uncoupling action of 3HTA and 3HPA. We verified that 3HTA and 3HPA (100 μM), but not 3HTDA (100 μM), strongly dissipated ΔΨm (Fig. 3E).

It was also observed that low concentrations of 3HTA and 3HPA (10–30 μM) provoked a strongly dissipation of ΔΨm when liver mitochondria were challenged by Ca^{2+} in a medium with low EGTA concentration (Fig. 3A: [$F_{(3,8)}$ = 189.1, P < 0.001]; 3B: [$F_{(3,8)}$ = 60.78, P < 0.001]). Furthermore, RR, an inhibitor of mitochondrial Ca^{2+} uptake, abolished the reduction of ΔΨm caused by 3HTA, emphasizing the importance of Ca^{2+} in this effect. It can be also observed that the classical inhibitors of MPT, CsA and ADP also protected glutamate plus malate (Fig. 3C: [$F_{(5,12)}$ = 57.81, P < 0.001]) or succinate-supported (Fig. 3D: [$F_{(4,10)}$ = 345.7, P < 0.001]) mitochondria against membrane depolarization. In contrast, it can be seen in the figure that DTT, NEM and GDP were not able to prevent the 3HTA-induced ΔΨm reduction, making unlikely an oxidative attack on thiol groups of the MPT pore (Fig. 3C and D). Finally, we found that the dicarboxylic 3HTDA did not alter ΔΨm in the presence or absence of Ca^{2+} (Fig. 3A and E). Similar results were obtained when using brain mitochondria in the same experimental conditions, although with less intense effects of 3HTA (Fig. 3F: [$F_{(4,10)}$ = 302.4, P < 0.001]).

3.3. Long-chain monocarboxylic 3-hydroxylated fatty acids decrease NAD(P)H matrix mitochondrial content in liver

In the next set of experiments we evaluated the effect of 3HTDA, 3HTA and 3HPA on mitochondrial NAD(P)H content in liver mitochondria supported by glutamate plus malate or succinate. We observed that 3HTA and 3HPA (100 μM), but not 3HTDA (100 μM), provoked a significant decrease of NAD(P)H levels (Fig. 4D).

In addition, 3HTA and 3HPA (30 μM) provoked a decrease of NAD(P)H content when Ca^{2+} was added to the medium that contained a low concentration of EGTA (Fig. 4A: [$F_{(3,8)}$ = 687.5, P < 0.001]), suggesting that the reduced equivalents were oxidized or lost from the matrix. Since the decrease in mitochondrial NAD(P)H content was prevented by CsA, ADP and RR, it is conceivable that this effect may be

attributed to MPT pore opening (Fig. 4B: [$F_{(3,8)}$ = 156.7, P < 0.001]; Fig. 4C: [$F_{(3,8)}$ = 137.7, P < 0.001]). Furthermore, 3HTDA, the dicarboxylic analogue of 3HTA, did not change NAD(P)H content both in the presence or absence of Ca^{2+} (Fig. 4A and Fig. 4D).

3.4. Long-chain monocarboxylic 3-hydroxylated fatty acids induce swelling in liver mitochondria

Considering that induction of MPT is classically accompanied by mitochondrial swelling, we investigated the influence of 3HTDA, 3HTA and 3HPA on mitochondrial swelling in Ca^{2+}-loaded mitochondria. It was found that 30 μM of 3HTA and 3HPA significantly induced swelling in liver mitochondria supported by glutamate plus malate in the presence of Ca^{2+} (Fig. 5A: [$F_{(3,8)}$ = 1072.0, P < 0.001]; Fig. 5B: [$F_{(3,8)}$ = 40.39, P < 0.001]). Furthermore, this effect was fully prevented by RR, CsA and ADP (Fig. 5B), indicating MPT induction by these LCHFA. In contrast, 3HTDA did not alter this parameter (Fig. 5A).

3.5. Long-chain monocarboxylic 3-hydroxylated fatty acids reduce Ca^{2+} retention capacity in liver mitochondria

Since MPT induction may compromise mitochondrial Ca^{2+} homeostasis and lead to release of this cation from the mitochondria, we determined the mitochondrial Ca^{2+} retention capacity in the presence of 3HTDA, 3HTA or 3HPA using glutamate plus malate or succinate as substrates. It can be seen in Fig. 6A–D that 3HTA and 3HPA (10–30 μM) significantly reduced the mitochondrial Ca^{2+} retention capacity, an effect
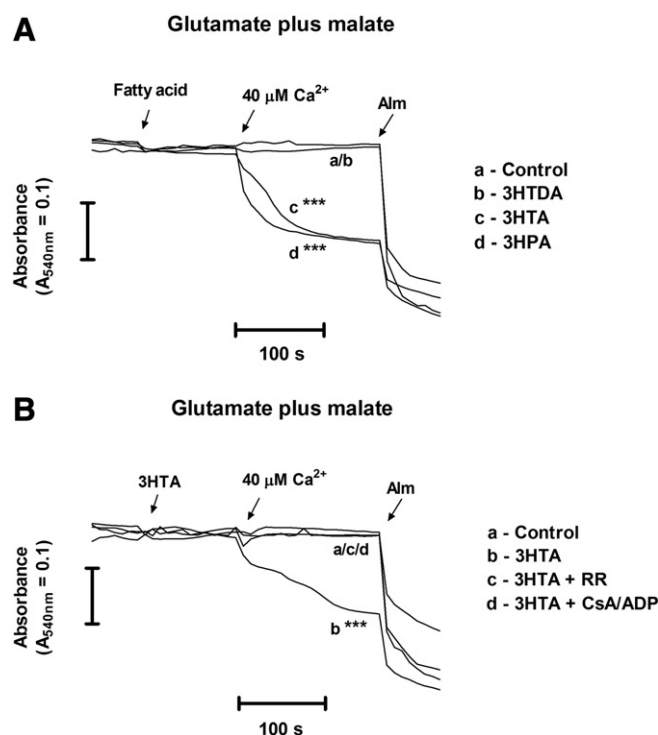
Fig. 5. Effects of 3-hydroxytetracanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial swelling in the presence of Ca^{2+}. All experiments were performed in a reaction media containing mitochondrial preparations (0.5 mg protein·mL^{-1}) supported by glutamate/malate. (A) 3HTDA, 3HTA or 3HPA (30 μM, lines b–d) was added 50 s after the beginning of the assay. (B) 3HTA (30 μM, lines b–d), ruthenium red (RR, 5 μM, line c) and cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line d) were added in the beginning of the assay. Controls (lines a) were performed in the absence of fatty acids. All traces refer to mitochondrial preparations supplemented by 40 μM Ca^{2+} 150 s after the beginning of the assay, as indicated. Alamethicin (Alm, 40 μg/mg of protein) was added at the end of the measurements. Traces are representative of three independent experiments (animals). ***P < 0.001, compared to controls (Duncan's multiple range test).

that was completely prevented by CsA. In contrast, 3HTDA did not alter mitochondrial Ca^{2+} retention capacity.

4. Discussion

We demonstrated here that the major monocarboxylic LCHFA accumulating in LCHAD deficiency (3HTA and 3HPA) disrupt mitochondrial functions in liver of young rats at concentrations as low as 10 μM, in contrast to the dicarboxylate fourteen carbon chain analogue 3HTDA, which caused no effect on all evaluated parameters. 3HPA and 3HTA impaired liver mitochondrial bioenergetics by behaving as uncouplers, metabolic inhibitors and MPT inductors. Mitochondrial Ca^{2+} retention capacity was also compromised by these fatty acids, impairing therefore cellular Ca^{2+} homeostasis.

We first observed a decrease in state 3 and uncoupled mitochondrial respiration caused by 3HTA and 3HPA indicating that these fatty acids may act as metabolic inhibitors. 3HTA and 3HPA also increased state 4 respiration and decreased RCR, ΔΨm and mitochondrial NAD(P)H content, implying that they also behave as uncouplers of oxidative phosphorylation. These observations may possibly be related to the lactic acidemia and mitochondrial morphological abnormalities observed in tissues from patients affected by LCHAD deficiency [13,32]. We also found that the ANT inhibitor ATC attenuated 3HTA-induced increase of resting respiration (state 4), suggesting that ANT was involved in this effect. In contrast, the UCP inhibitor GDP did not change this effect, ruling out a role for UCP in the increase of resting respiration in the liver. It is of note that UCP is poorly expressed in liver mitochondria and its low expression in this tissue may possibly explain the reason by which GDP did not prevent the effects elicited by the LCHFA [33]. However, considering that GDP was also unable to prevent the 3HTA-induced reduction of membrane potential in brain mitochondria, which express UCPs [33], it is conceivable that these UCPs were not involved in the effects elicited by the LCHFA.

Uncoupling of oxidative phosphorylation had been previously demonstrated for non-hydroxylated LCFA. The protonophoric mechanism of this effect is thought to be due to the transbilayer movement of undissociated (linked to protons) fatty acids through the mitochondrial inner membrane towards the mitochondrial matrix. The passage of dissociated fatty acids in the opposite direction to the intermembrane mitochondrial space is usually facilitated by ANT and UCP [34–37]. Although our findings demonstrated that ANT is involved in the uncoupling effects of 3HTA and 3HPA, we cannot discard that LCFA acid anions may also be transferred by other mitochondrial carriers, such as the glutamate/aspartate antiporter [38,39], the mono- and tricarboxylate carriers [40] and the phosphate carrier [41].

Furthermore, 3HTA and 3HPA (10–30 μM), but not 3HTDA, strongly dissipated ΔΨm in the presence of Ca^{2+}. Furthermore, RR, a known inhibitor of the mitochondrial Ca^{2+} uniporter [42], and CsA plus ADP, classical inhibitors of MPT, prevented ΔΨm dissipation, supporting a role for Ca^{2+} and MPT induction in these effects. CsA is an inhibitor of MPT by inactivating the cyclophilin D, a mitochondrial matrix protein associated with the MPT occurrence [43–46], whereas ADP is a potent inhibitor of MPT in isolated mitochondria by binding to ANT in the matrix side [31,47]. We also found that neither the potent reducing agent DTT nor the thiol group protector NEM avoided the ΔΨm loss, implying that an oxidative attack to MPT pore was probably not involved in 3HTA-induced MPT. In this regard, a common explanation for the induction of nonselective permeabilization was the oxidation of membrane protein thiol groups on the MPT pore [48,49], but this was not the case for these compounds, not supporting therefore a role for oxidative damage in this effect.

3HTA and 3HPA (30 μM) also decreased mitochondrial matrix NAD(P)H content after Ca^{2+} loading, which was totally abolished by CsA plus ADP and by RR, implying that the decrease of matrix reduced equivalents may have occurred as a consequence of nonselective inner membrane permeabilization due to MPT. Alternatively, NADH consumption due to MPT induction and consequent activation of the electron transport flow may have also contributed to the reduction of NAD(P)H concentrations as observed here [48,50,51].

The nonselective permeabilization caused by MPT activation is usually accompanied by swelling and loss of mitochondrial elements (Ca^{2+}, Mg^{2+}, glutathione, NADH and NADPH), including pro-apoptotic factors
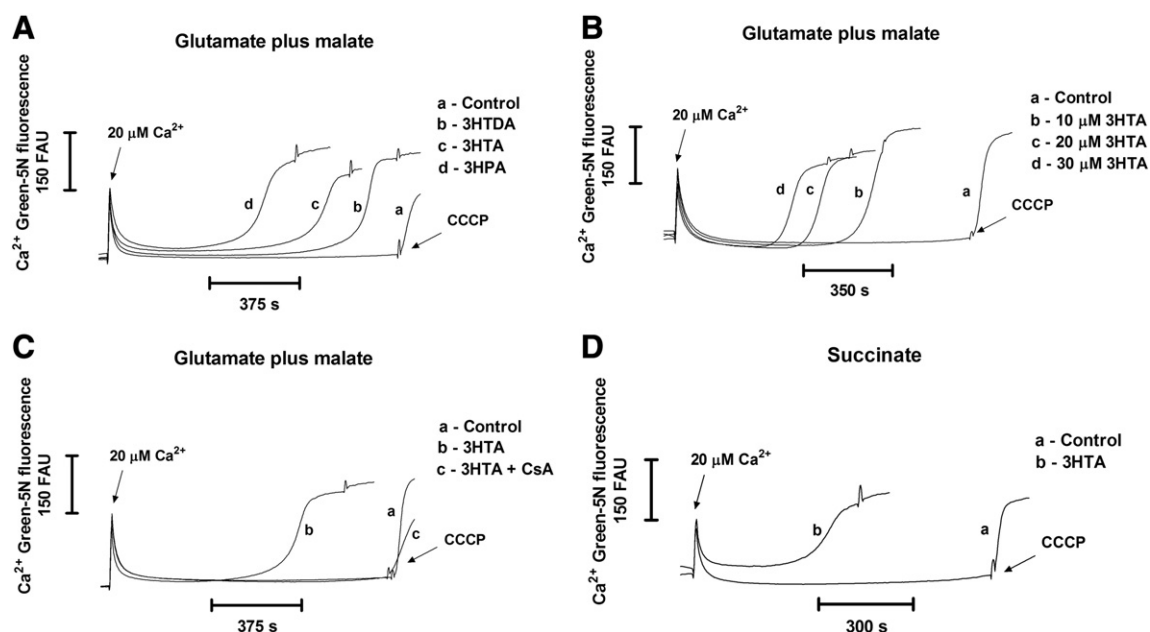

Fig. 6. Effects of 3-hydroxytetradecanodioic (3HTDA), 3-hydroxytetradecanoic (3HTA) and 3-hydroxypalmitic (3HPA) acids on mitochondrial Ca^{2+} retention capacity. All experiments were performed in a reaction media containing mitochondrial preparations (0.5 mg protein·mL^{-1}) supported by glutamate/malate or succinate. (A) 3HTDA, 3HTA or 3HPA (30 μM, lines b–d) was added in the beginning of the assay. (B) 3HTA (10–30 μM, lines b–d), (C) 3HTA (30 μM, lines b–c) and cyclosporin A (CsA, 1 μM, line c) were added in the beginning of the assay. (D) 3HTA (30 μM, lines b). Controls (lines a) were performed in the absence of fatty acids. All traces refer to mitochondrial preparations supplemented by 20 μM Ca^{2+} 50 s after the beginning of the assay, as indicated. CCCP (3 μM) was added in the end of the assays. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU).

such as cytochrome c, potentially leading to apoptosis or/and necrosis, as well as impairment of oxidative phosphorylation and ATP synthesis [52–58]. In this regard, we demonstrated that 3HTA and 3HPA provoked extensive mitochondrial swelling after Ca^{2+} addition and this was totally prevented by CsA and ADP, as well as by RR, confirming the occurrence of MPT.

Another important contribution of our work was that the major fatty acids accumulated in LCHAD deficiency strongly compromised the capacity of mitochondria to uptake and retain Ca^{2+}, which is a crucial function of this organelle [59–63]. Again, this effect was totally prevented by CsA, indicating that the reduction in mitochondrial Ca^{2+} retention capacity was a consequence of MPT induction. Indeed, MPT pore opening could allow Ca^{2+} release from the matrix after reaching a threshold, overcoming mitochondrial Ca^{2+} retention capacity [52,55,57].

It is of interest that the effects found in our work were selective for the monocarboxylic LCHFA, since the dicarboxylic 3HTDA, a structurally analogue of 3HTA, did not alter any of the tested parameters. Noteworthy, previous unpublished findings from our laboratory showed that the dicarboxylic acids adipic, suberic and sebacic acids do not alter important parameters of mitochondrial bioenergetics. A possible explanation for the lack of effect of the dicarboxylates could be because they cannot enter into mitochondrial matrix through the inner mitochondrial membrane by diffusion, as possibly occurs with the monocarboxylic analogues. In this context, monocarboxylic LCFA are in general good protonophores (unouplers), in contrast to dicarboxylic LCFA [64]. Furthermore, it was postulated that the monocarboxylic LCFA have the ability to undergo flip–flop movements in the phospholipid core of the inner mitochondrial membrane and to be transported back to the intermembrane space by mitochondrial anion carriers in the anionic form [65].

Our present work shows that the significant findings observed in liver mitochondria were more pronounced and achieved with lower doses of the LCHFA, as compared to brain mitochondria [24,26], indicating that liver is more vulnerable to the deleterious effects of these fatty acids. It is therefore tempting to speculate that the higher vulnerability of the liver to the effects of 3HTA and 3HPA on mitochondrial functions may be possibly associated with the severe liver symptomatology, as compared to the neurological manifestations, in patients with LCHAD deficiency [11,14]. On the other hand, considering that fatty acid synthesis and metabolism is very active in the liver, and besides that this tissue essentially uses fatty acids for energy supply, it is conceivable that the concentrations of these monocarboxylic hydroxylated fatty acids in patients affected by LCHAD deficiency are much higher in the liver, relatively to other tissues including the brain.

Regarding the mechanisms of LCHFA-induced MPT, it may have occurred due to the uncoupling effects of 3HTA and 3HPA, which were previously shown to provoke MPT pore opening [66–69]. MPT induction could be also consequent of the interaction of these fatty acids with the cytosolic side of ANT, as previously demonstrated for ATC and other similar compounds [70–72] since ATC attenuated state 4 increase.

We cannot at the present determine whether 3HTA and 3HPA bind to albumin in the liver, since to the best of our knowledge there is no work in the literature describing the interaction of long-chain hydroxylated fatty acid with albumin in various tissues. However, it is important to emphasize that we used low albumin concentrations in the medium similar to those found in liver cytosol to simulate an intracellular environment [73]. Our results obtained under these experimental conditions with low albumin concentration showed that 3HTA and 3HPA caused significant effects on the evaluated parameters, suggesting that these fatty acids disturb mitochondrial energy homeostasis which may be attributed to their free active unbinding form.

At the present it is difficult to establish the pathophysiological relevance of our data since the liver concentrations of free LCHFA in patients affected by LCHAD deficiency are still unknown. However, it is conceivable that they may contribute to explain the pathophysiology of this disease since under experimental conditions that simulate an intracellular environment, the LCHFA that most accumulate in this disorder, particularly 3HPA and 3HTA, reduced the $\Delta\Psi m$ and provoked mitochondrial swelling in the presence of calcium that were prevented by CsA, ADP and RR at similar or even lower concentrations (e.g.,: 10 μM) than those found in plasma of the affected patients, especially during crises of metabolic decompensation that are accompanied by a worsening of hepatic clinical manifestations [3,74]. It is emphasized that these crises generally follow infections and accelerated catabolism that leads to increased tissue levels of these fatty acids [74].

5. Conclusions

In conclusion, to the best of our knowledge we provide for the first time experimental evidence that the monocarboxylic LCHFA that most accumulate in LCHAD deficiency behave as strong metabolic inhibitors, uncoupling agents and MPT inductors in the liver from young rats. Since these effects might potentially reduce energy production, we propose that disruption of mitochondrial homeostasis may be associated with the hepatic failure presented by patients affected by LCHAD deficiency.

Transparency document

The Transparency document associated with this article can be found, in the online version.

Acknowledgements

References

[1] R.J. Wanders, M. Duran, L. Ijlst, J.P. de Jager, A.H. van Gennip, C. Jakobs, L. Dorland, F.J. van Sprang, Sudden infant death and long-chain 3-hydroxyacyl-CoA dehydrogenase, Lancet 2 (1989) 52–53.

[2] C.R. Scriver, A.L. Beaudet, W.S. Sly, D. Valle, The Metabolic and Molecular Bases of Inherited Disease, 8 ed. McGraw-Hill Inc., New York, 2001.

[3] C.G. Costa, L. Dorland, I.T. de Almeida, C. Jakobs, M. Duran, B.T. Poll-The, The effect of fasting, long-chain triglyceride load and carnitine load on plasma long-chain acylcarnitine levels in mitochondrial very long-chain acyl-CoA dehydrogenase deficiency, J. Inherit. Metab. Dis. 21 (1998) 391–399.

[4] S.R. Hintz, D. Matern, A. Strauss, M.J. Bennett, H.E. Hoyme, S. Schelley, J. Kobori, C. Colby, N.L. Lehman, G.M. Enns, Early neonatal diagnosis of long-chain 3-hydroxyacyl coenzyme a dehydrogenase and mitochondrial trifunctional protein deficiencies, Mol. Genet. Metab. 75 (2002) 120–127.

[5] S.E. Olpin, S. Clark, B.S. Andresen, C. Bischoff, R.K. Olsen, N. Gregersen, A. Chakrapani, M. Downing, N.J. Manning, M. Sharrard, J.R. Bonham, F. Muntoni, D.N. Turnbull, M. Pourfarzam, Biochemical, clinical and molecular findings in LCHAD and general mitochondrial trifunctional protein deficiency, J. Inherit. Metab. Dis. 28 (2005) 533–544.

[6] J. Sander, S. Sander, U. Steuerwald, N. Janzen, M. Peter, R.J. Wanders, I. Marquardt, G.C. Korenke, A.M. Das, Neonatal screening for defects of the mitochondrial trifunctional protein, Mol. Genet. Metab. 85 (2005) 108–114.

[7] E. Riudor, Neonatal onset in fatty acid oxidation disorders: how can we minimize morbidity and mortality? J. Inherit. Metab. Dis. 21 (1998) 619–623.

[8] F.V. Ventura, J.P. Ruiter, I.J. L, I.T. de Almeida, R.J. Wanders, Lactic acidosis in long-chain fatty acid beta-oxidation disorders, J. Inherit. Metab. Dis. 21 (1998) 645–654.

[9] B. Wilcken, Fatty acid oxidation disorders: outcome and long-term prognosis, J. Inherit. Metab. Dis. 33 (2010) 501–506.

[10] S.E. Waisbren, Y. Landau, J. Wilson, J. Vockley, Neuropsychological outcomes in fatty acid oxidation disorders: 85 cases detected by newborn screening, Dev. Disabil. Res. Rev. 17 (2013) 260–268.

[11] D. Moczulski, I. Majak, D. Mamczur, An overview of beta-oxidation disorders, Postepy Hig. Med. Dosw. (Online) 63 (2009) 266–277.

[12] F. Rocchiccioli, R.J. Wanders, P. Aubourg, C. Vianey-Liaud, L. Ijlst, M. Fabre, N. Cartier, P.F. Bougneres, Deficiency of long-chain 3-hydroxyacyl-CoA dehydrogenase: a cause of lethal myopathy and cardiomyopathy in early childhood, Pediatr. Res. 28 (1990) 657–662.

[13] R.H. Amirkhan, C.F. Timmons, K.O. Brown, M.J. Weinberger, M.J. Bennett, Clinical, bio-chemical, and morphologic investigations of a case of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, Arch. Pathol. Lab. Med. 121 (1997) 730–734.

[14] T. Tyni, J. Rapola, A. Paetau, A. Palotie, H. Pihko, Pathology of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency caused by the G1528C mutation, Pediatr. Pathol. Lab. Med. 17 (1997) 427–447.

[15] M. Gillingham, S. Van Calcar, D. Ney, J. Wolff, C. Harding, Dietary management of long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (LCHADD). A case report and survey, J. Inherit. Metab. Dis. 22 (1999) 123–131.

[16] J.M. Saudubray, D. Martin, P. de Lonlay, G. Touati, F. Poggi-Travert, D. Bonnet, P. Jouvet, M. Boutron, A. Slama, C. Vianey-Saban, J.P. Bonnefont, D. Rabier, P. Kamoun, M. Brivet, Recognition and management of fatty acid oxidation defects: a series of 107 patients, J. Inherit. Metab. Dis. 22 (1999) 488–502.

[17] U. Spiekerkoetter, P.A. Wood, Mitochondrial fatty acid oxidation disorders: patho-physiological studies in mouse models, J. Inherit. Metab. Dis. 33 (2010) 539–546.

[18] C.B. Haglind, M.H. Stenlid, S. Ask, J. Alm, A. Nemeth, U. Dobeln, A. Nordenstrom, Growth in long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, JIMD Rep. 8 (2013) 81–90.

[19] U. Spiekerkoetter, M.J. Bennett, B. Ben-Zeev, A.W. Strauss, I. Tein, Peripheral neurop-athy, episodic myoglobinuria, and respiratory failure in deficiency of the mitochon-drial trifunctional protein, Muscle Nerve 29 (2004) 66–72.

[20] U. Spiekerkoetter, Z. Khuchua, Z. Yue, M.J. Bennett, A.W. Strauss, General mitochon-drial trifunctional protein (TFP) deficiency as a result of either alpha- or beta-subunit mutations exhibits similar phenotypes because mutations in either subunit alter TFP complex expression and subunit turnover, Pediatr. Res. 55 (2004) 190–196.

[21] A. Sperk, M. Mueller, U. Spiekerkoetter, Outcome in six patients with mitochondrial trifunctional protein disorders identified by newborn screening, Mol. Genet. Metab. 101 (2010) 205–207.

[22] R.K. Olsen, N. Cornelius, N. Gregersen, Genetic and cellular modifiers of oxidative stress: what can we learn from fatty acid oxidation defects? Mol. Genet. Metab. 110 (Suppl.) (2013) S31–S39.

[23] T. Tyni, A. Majander, H. Kalimo, J. Rapola, H. Pihko, Pathology of skeletal muscle and impaired respiratory chain function in long-chain 3-hydroxyacyl-CoA dehydroge-nase deficiency with the G1528C mutation, Neuromuscul. Disord. 6 (1996) 327–337.

[24] A.M. Tonin, G.C. Ferreira, M. Grings, C.M. Viegas, E.N. Busanello, A.U. Amaral, A. Zanatta, P.F. Schuck, M. Wajner, Disturbance of mitochondrial energy homeostasis caused by the metabolites accumulating in LCHAD and MTP deficiencies in rat brain, Life Sci. 86 (2010) 825–831.

[25] A.M. Tonin, A.U. Amaral, E.N. Busanello, M. Grings, R.F. Castilho, M. Wajner, Long-chain 3-hydroxy fatty acids accumulating in long-chain 3-hydroxyacyl-CoA dehy-drogenase and mitochondrial trifunctional protein deficiencies uncouple oxidative phosphorylation in heart mitochondria, J. Bioenerg. Biomembr. 45 (2013) 47–57.

[26] A.M. Tonin, A.U. Amaral, E.N. Busanello, J. Gasparotto, D.P. Gelain, N. Gregersen, M. Wajner, Mitochondrial bioenergetics deregulation caused by long-chain 3-hydroxy fatty acids accumulating in LCHAD and MTP deficiencies in rat brain: a possible role of mPTP opening as a pathomechanism in these disorders? Biochim. Biophys. Acta 1842 (2014) 1658–1667.

[27] S.R. Mirandola, D.R. Melo, P.F. Schuck, G.C. Ferreira, M. Wajner, R.F. Castilho, Methylmalonate inhibits succinate-supported oxygen consumption by interfering with mitochondrial succinate uptake, J. Inherit. Metab. Dis. 31 (2008) 44–54.

[28] M.M. Bradford, A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein–dye binding, Anal. Biochem. 72 (1976) 248–254.

[29] K.E. Akerman, M.K. Wikstrom, Safranine as a probe of the mitochondrial membrane potential, FEBS Lett. 68 (1976) 191–197.

[30] T.R. Figueira, D.R. Melo, A.E. Vercesi, R.F. Castilho, Safranine as a fluorescent probe for the evaluation of mitochondrial membrane potential in isolated organelles and permeabilized cells, Methods Mol. Biol. 810 (2012) 103–117.

[31] A. Saito, R.F. Castilho, Inhibitory effects of adenine nucleotides on brain mitochon-drial permeability transition, Neurochem. Res. 35 (2010) 1667–1674.

[32] T. Tyni, H. Pihko, Long-chain 3-hydroxyacyl-CoA dehydrogenase deficiency, Acta Paediatr. 88 (1999) 237–245.

[33] J. Nedergaard, D. Ricquier, L.P. Kozak, Uncoupling proteins: current status and therapeutic prospects, EMBO Rep. 6 (2005) 917–921.

[34] P. Schonfeld, L. Schild, W. Kunz, Long-chain fatty acids act as protonophoric uncouplers of oxidative phosphorylation in rat liver mitochondria, Biochim. Biophys. Acta 977 (1989) 266–272.

[35] P. Schonfeld, Anion permeation limits the uncoupling activity of fatty acids in mitochondria, FEBS Lett. 303 (1992) 190–192.

[36] F. Goglia, V.P. Skulachev, A function for novel uncoupling proteins: antioxidant defense of mitochondrial matrix by translocating fatty acid peroxides from the inner to the outer membrane leaflet, FASEB J. 17 (2003) 1585–1591.

[37] C. Affourtit, P.G. Crichton, N. Parker, M.D. Brand, Novel uncoupling proteins, Novartis Found. Symp. 287 (2007) 70–80 (discussion 80–91).

[38] V.P. Skulachev, Uncoupling: new approaches to an old problem of bioenergetics, Biochim. Biophys. Acta 1363 (1998) 100–124.

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

[40] P. Schonfeld, M.R. Wieckowski, L. Wojtczak, Long-chain fatty acid-promoted swell-ing of mitochondria: further evidence for the protonophoric effect of fatty acids in the inner mitochondrial membrane, FEBS Lett. 471 (2000) 108–112.

[41] M. Zackova, R. Kramer, P. Jezek, Interaction of mitochondrial phosphate carrier with fatty acids and hydrophobic phosphate analogs, Int. J. Biochem. Cell Biol. 32 (2000) 499–508.

[42] C.L. Moore, Specific inhibition of mitochondrial Ca^{++} transport by ruthenium red, Biochim. Biophys. Res. Commun. 42 (1971) 298–305.

[43] A. Tanveer, S. Virji, L. Andreeva, N.F. Totty, J.J. Hsuan, J.M. Ward, M. Crompton, Involvement of cyclophilin in the activation of a mitochondrial pore by Ca^{2+} and oxidant stress, Eur. J. Biochem. 238 (1996) 166–172.

[44] C.P. Baines, R.A. Kaiser, N.H. Purcell, N.S. Blair, H. Osinska, M.A. Hambleton, E.W. Brunskill, M.R. Sayen, R.A. Gottlieb, G.W. Dorn, J. Robbins, J.D. Molkentin, Loss of cyclophilin D reveals a critical role for mitochondrial permeability transition in cell death, Nature 434 (2005) 658–662.

[45] E. Basso, L. Fante, J. Fowlkes, V. Petronilli, M.A. Forte, P. Bernardi, Properties of the permeability transition pore in mitochondria devoid of cyclophilin D, J. Biol. Chem. 280 (2005) 18558–18561.

[46] P. Bernardi, The mitochondrial permeability transition pore: a mystery solved? Front. Physiol. 4 (2013) 95.

[47] H. Rottenberg, M. Marbach, Adenine nucleotides regulate Ca^{2+} transport in brain mitochondria, FEBS Lett. 247 (1989) 483–486.

[48] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Mitochondrial permeability transition and oxidative stress, FEBS Lett. 495 (2001) 12–15.

[49] A.J. Kowaltowski, A.E. Vercesi, R.F. Castilho, Mitochondrial membrane protein thiol re-activity with N-ethylmaleimide or mersalyl is modified by Ca^{2+}: correlation with mito-chondrial permeability transition, Biochim. Biophys. Acta 1318 (1997) 395–402.

[50] D. Le-Quoc, K. Le-Quoc, Relationships between the NAD(P) redox state, fatty acid oxidation, and inner membrane permeability in rat liver mitochondria, Arch. Biochem. Biophys. 273 (1989) 466–478.

[51] J.P. Kehrer, L.G. Lund, Cellular reducing equivalents and oxidative stress, Free Radic. Biol. Med. 17 (1994) 65–75.

[52] M. Zoratti, I. Szabo, The mitochondrial permeability transition, Biochim. Biophys. Acta 1241 (1995) 139–176.

[53] A.A. Starkov, The molecular identity of the mitochondrial Ca^{2+} sequestration system, FEBS J. 277 (2010) 3652–3663.

[54] A. Rasola, P. Bernardi, Mitochondrial permeability transition in Ca(2+)-dependent apoptosis and necrosis, Cell Calcium 50 (2011) 222–233.

[55] P. Bernardi, S. von Stockum, The permeability transition pore as a Ca(2+) release channel: new answers to an old question, Cell Calcium 52 (2012) 22–27.

[56] R. Rizzuto, D. De Stefani, A. Raffaello, C. Mammucari, Mitochondria as sensors and regulators of calcium signalling, Nat. Rev. Mol. Cell Biol. 13 (2012) 566–578.

[57] M. Crompton, S. Virji, V. Doyle, N. Johnson, J.M. Ward, The mitochondrial permeabil-ity transition pore, Biochem. Soc. Symp. 66 (1999) 167–179.

[58] T.R. Figueira, M.H. Barros, A.A. Camargo, R.F. Castilho, J.C. Ferreira, A.J. Kowaltowski, F.E. Sluse, N.C. Souza-Pinto, A.E. Vercesi, Mitochondria as a source of reactive oxygen and nitrogen species: from molecular mechanisms to human health, Antioxid. Redox Signal. 18 (2013) 2029–2074.

[59] J.M. Baughman, F. Perocchi, H.S. Girgis, M. Plovanich, C.A. Belcher-Timme, Y. Sancak, X.R. Bao, L. Strittmatter, O. Goldberger, R.L. Bogorad, V. Koteliansky, V.K. Mootha, In-tegrative genomics identifies MCU as an essential component of the mitochondrial calcium uniporter, Nature 476 (2011) 341–345.

[60] D. De Stefani, A. Raffaello, E. Teardo, I. Szabo, R. Rizzuto, A forty-kilodalton protein of the inner membrane is the mitochondrial calcium uniporter, Nature 476 (2011) 336–340.

[61] X. Pan, J. Liu, T. Nguyen, C. Liu, J. Sun, Y. Teng, M.M. Fergusson, I.I. Rovira, M. Allen, D.A. Springer, A.M. Aponte, M. Gucek, R.S. Balaban, E. Murphy, T. Finkel, The physi-ological role of mitochondrial calcium revealed by mice lacking the mitochondrial calcium uniporter, Nat. Cell Biol. 15 (2013) 1464–1472.

[62] S. Marchi, P. Pinton, The mitochondrial calcium uniporter complex: molecular compo-nents, structure and physiopathological implications, J. Physiol. 592 (2014) 829–839.

[63] D. Pendin, E. Greotti, T. Pozzan, The elusive importance of being a mitochondrial Ca(2+) uniporter, Cell Calcium 55 (2014) 139–145.

[64] L. Wojtczak, M.R. Wieckowski, P. Schonfeld, Protonophoric activity of fatty acid analogs and derivatives in the inner mitochondrial membrane: a further argument for the fatty acid cycling model, Arch. Biochem. Biophys. 357 (1998) 76–84.

[65] V.P. Skulachev, Fatty acid circuit as a physiological mechanism of uncoupling of oxidative phosphorylation, FEBS Lett. 294 (1991) 158–162.

[66] P. Bernardi, Modulation of the mitochondrial cyclosporin A-sensitive permeability transition pore by the proton electrochemical gradient. Evidence that the pore can be opened by membrane depolarization, J. Biol. Chem. 267 (1992) 8834–8839.

[67] R.F. Castilho, J.A. Vicente, A.J. Kowaltowski, A.E. Vercesi, 4,6-Dinitro-o-cresol uncou-ples oxidative phosphorylation and induces membrane permeability transition in rat liver mitochondria, Int. J. Biochem. Cell Biol. 29 (1997) 1005–1011.

[68] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Opening of the mitochondrial perme-ability transition pore by uncoupling or inorganic phosphate in the presence of Ca^{2+} is dependent on mitochondrial-generated reactive oxygen species, FEBS Lett. 378 (1996) 150–152.

[69] E.B. Zago, R.F. Castilho, A.E. Vercesi, The redox state of endogenous pyridine nucleo-tides can determine both the degree of mitochondrial oxidative stress and the solute selectivity of the permeability transition pore, FEBS Lett. 478 (2000) 29–33.

[70] P. Schonfeld, R. Bohnensack, Fatty acid-promoted mitochondrial permeability transition by membrane depolarization and binding to the ADP/ATP carrier, FEBS Lett. 420 (1997) 167–170.

[71] M.R. Wieckowski, L. Wojtczak, Fatty acid-induced uncoupling of oxidative phos-phorylation is partly due to opening of the mitochondrial permeability transition pore, FEBS Lett. 423 (1998) 339–342.

[72] M. Di Paola, P. Zaccagnino, C. Oliveros-Celis, M. Lorusso, Arachidonic acid induces specific membrane permeability increase in heart mitochondria, FEBS Lett. 580 (2006) 775–781.

[73] E. Baraona, M.A. Leo, S.A. Borowsky, C.S. Lieber, Pathogenesis of alcohol-induced accumulation of protein in the liver, J. Clin. Invest. 60 (1977) 546–554.

[74] L. Hagenfeldt, U. von Dobeln, E. Holme, J. Alm, G. Brandberg, E. Enocksson, L. Lindeberg, 3-Hydroxydicarboxylic aciduria—a fatty acid oxidation defect with severe prognosis, J. Pediatr. 116 (1990) 387–392.