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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?

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ABSTRACT

Long-chain 3-hydroxylated fatty acids (LCHFA) accumulate in long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies. Affected patients usually present severe neonatal symptoms involving cardiac and hepatic functions, although long-term neurological abnormalities are also commonly observed. Since the underlying mechanisms of brain damage are practically unknown and have not been properly investigated, we studied the effects of LCHFA on important parameters of mitochondrial homeostasis in isolated mitochondria from cerebral cortex of developing rats. 3-Hydroxytetradecanoic acid (3 HTA) reduced mitochondrial membrane potential, NAD(P)H levels, Ca^{2+} retention capacity and ATP content, besides inducing swelling, cytochrome c release and H_2O_2 production in Ca^{2+}-loaded mitochondrial preparations. We also found that cyclosporine A plus ADP, as well as ruthenium red, a Ca^{2+} uptake blocker, prevented these effects, suggesting the involvement of the mitochondrial permeability transition pore (mPTP) and an important role for Ca^{2+}, respectively. 3-Hydroxydodecanoic and 3-hydroxypalmitic acids, that also accumulate in LCHAD and MTP deficiencies, similarly induced mitochondrial swelling and decreased ATP content, but to a variable degree pending on the size of their carbon chain. It is proposed that mPTP opening induced by LCHFA disrupts brain bioenergetics and may contribute at least partly to explain the neurologic dysfunction observed in patients affected by LCHAD and MTP deficiencies.

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1. Introduction

Long-chain 3-hydroxy-acyl-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 [1–3]. Mutations in the genes that encode the MTP complex can lead to reduction or absent activity of all MTP complex enzymes or isolated LCHAD and LCKT deficiencies, being LCHAD deficiency the most frequent disorder [1,3,4].

LCHAD and MTP deficiencies are recognized as severe and life-threatening diseases what makes an early identification and treatment essential to improve patient survival. Both disorders are undistinguishable clinically and biochemically, presenting with a multiorgan involvement and elevated levels of LCFA and their hydroxylated derivatives (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. Furthermore, affected patients can also present later in life irreversible peripheral neuropathy and retinopathy [5], as well as speech and developmental delay, hypotonia, and lethargy that indicate cerebral dysfunction [6,7]. Since hypoglycemia is commonly found in these individuals, poor glucose uptake could possibly underlie these neurologic symptoms. However, we cannot rule out that the long-term neurologic symptomatology may be associated with the toxicity of LCHFA or their 3-hydroxy-long-chain acylcarnitines that accumulate in these disorders [5,8–10]. Although the exact pathomechanisms underlying the toxic effects of the LCHFA are poorly understood, recent studies demonstrated induction of oxidative stress in skin fibroblasts from MTP deficient patients [11]. It has been also demonstrated that LCHFA induce lipid and protein oxidative damage and decrease the antioxidant defenses in rat cerebral cortex *in vitro* [12]. Impairment of mitochondrial respiration and decrease of reduced equivalents caused by LCHFA, probably secondary to uncoupling of oxidative phosphorylation, were also shown in forebrain and heart of adolescent rats [13,14]. These data provide evidence that LCHFA disturb energy and redox mitochondrial homeostasis in the brain and are in line with the observations that muscle biopsies from LCHAD deficient patients showed abnormal mitochondrial morphology with swollen appearance containing fat infiltration [15]. However, more work is necessary to ascertain in more details the exact role of LCHFA on brain bioenergetics.

In this context, it has been long believed that under physiological conditions there is only a minor utilization of long-chain fatty acids (LCFA) in adult brain for energy metabolism because these compounds do not penetrate into brain and cannot be oxidized to generate ATP. However, some studies indicate that normal brain functioning depends on these fatty acids that are obtained from the blood at all ages but particularly in developing mammals. Indeed, fatty acids, and especially essential fatty acids, are continually required by developing and adult mammalian brain, where they can be oxidized to CO_2, elongated, incorporated into complex lipids, or follow other routes [16–20]. On the other hand, although it has been proposed that LCFA would not be expected to pass through the blood brain barrier (BBB) because they bind tightly to plasma protein and they are almost completely ionized at pH 7.4, some works found that they dissociate very easily from albumin [21] and that saturated and unsaturated LCFA are rapidly transported through the BBB either by passive diffusion using a 'flip–flop' mechanism or by a protein-mediated transport mechanism using specific transport proteins (FATP) [22–25]. Furthermore, chronic dietary ingestion of a high content of saturated fatty acids and unsaturated fatty acids provokes oxidative damage in endothelial cells leading to BBB damage and dysfunction that could increase membrane permeability [26,27]. Finally, fatty acid binding proteins and carnitine have been found in the brain tissue, supporting a role for fatty acid metabolism in neurodevelopment [24,28].

Thus, in the present work we investigated the role of 3-hydroxydodecanoic (3 HDA), 3-hydroxytetradecanoic (3 HTA) and 3-hydroxypalmitic (3 HPA) acids on important parameters of mitochondrial homeostasis, such as membrane potential, swelling, Ca^{2+} retention capacity, NAD(P)H content, H_2O_2 production, cytochrome *c* release and ATP content in Ca^{2+}-loaded mitochondrial preparations from cerebral cortex from adolescent rats.

2. Material and methods

2.1. Reagents

All chemicals were purchased from Sigma-Aldrich (St. Louis, MO, USA). Stock solutions of a racemic mixture (DL) 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 and proved not to alter 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 an 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 [29], with slight modifications [30]. 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 2000 ×g for 3 min at 4 °C. The pellet was discarded and the supernatant was centrifuged at 12,000 ×g for 8 min at 4 °C. The resultant pellet was resuspended in 5 mL of isolation buffer containing 20 μL of 10% digitonin (final concentration of 0.04%), and centrifuged at 12,000 ×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 ×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 the method of Bradford [31] using BSA as standard. This preparation results in a mixture of synaptosomal and non-synaptosomal mitochondria similar to the general brain composition. Mitochondria obtained from cerebral cortex were used in the assays immediately after isolation and assays were carried out in the absence or presence of Ca^{2+}.

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 MgCl$_2$, 30 μM EGTA, 0.1 mg·mL^{-1} BSA, 5 mM HEPES, 2 mM KH$_2$PO$_4$, and pH 7.2, using mitochondria (0.5 mg protein·mL^{-1}) supported by 2.5 mM glutamate plus 2.5 mM malate. 3 HDA (10–30 μM), 3 HTA (10–60 μM), 3 HPA (10–30 μM), CaCl$_2$ (0–30 μM), CCCP (3 μM), antimycin A (AA, 0.1 μg·mL^{-1}) and alamethicin (Alm, 40 μg·mL^{-1}) were added as indicated by the arrows in the figures. In some experiments, ruthenium red (RR, 1 μM), cyclosporin A (CsA, 1 μM), ADP (300 μM) and 1 μg·mL^{-1} oligomycin A were added in the assay. Traces are representative of independent experiments carried out in mitochondrial preparations from cerebral cortex of three animals and were expressed as fluorescence arbitrary units (FAU), unless otherwise stated. Statistical analyses were also carried out by analyzing quantitatively the data obtained from the assays. Various blanks were used. Some blanks did not contain the LCHFA and served as controls, whereas others were devoid of brain mitochondrial preparations in the incubation medium and served to detect interferences (artifacts) of the tested metabolites on the techniques utilized to measure the mitochondrial parameters (results not shown).

2.5. Mitochondrial membrane potential

Mitochondrial membrane potential ($\Delta\Psi$m) was estimated according to Akerman and Wikstrom [32] and Kowaltowski et al. [33]. The fluorescence of 5 μM cationic dye safranin O, a $\Delta\Psi$m indicator, was followed at excitation and emission wavelengths of 495 and 586 nm. CCCP was added at the end of the measurements to abolish $\Delta\Psi$m.

2.6. Mitochondrial swelling

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

2.7. Mitochondrial Ca²⁺ retention capacity

Ca^{2+} retention capacity was determined following the external free Ca^{2+} levels using 0.2 μM Calcium Green-5 N (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 [34]. At the end of the measurements, maximal Ca^{2+} release was induced by CCCP.

2.8. Mitochondrial NAD(P)H

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

2.9. Mitochondrial hydrogen peroxide (H₂O₂) release

H$_2$O$_2$ production was assessed through the oxidation of Amliflu red (5 μM) in the presence of horseradish peroxidase (0.5 U/mL) [35]. 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 at the end of the experiments to provoke maximal H$_2$O$_2$ production. The quantification of the rates of H$_2$O$_2$ emission in pmol/min/mg protein was performed by creating a slope coefficient with addition of known amounts of H$_2$O$_2$.

2.10. Cytochrome c immunocontent

The swelling experiments were also carried out in the absence of Alm. The incubation medium was collected afterwards, and centrifuged at 12,000 $\times g$ for 10 min at 4 °C in order to sediment mitochondria. The resultant pellet was resuspended in 1 $\times$ RIPA buffer and centrifuged at 10,000 $\times g$ for 5 min 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 min at room temperature in SNAP i.d.® 2.0 Protein Detection System Merck Millipore (Billerica, MA, USA) with the mouse monoclonal anti-cytochrome *c* primary antibody (Abcam, Massachusetts, USA) (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 min in SNAP (1:5000 dilution range), and 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 production

Mitochondrial fractions (0.75 mg protein·mL^{-1}) were incubated in respiring medium containing 0.3 M sucrose, 5 mM MOPS, 5 mM KH$_2$PO$_4$, 30 μM EGTA, 0.1% BSA, and 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 $\times g$ for 5 min at 4 °C, 400 μL aliquots of the supernatant were neutralized with 100 μL of 1 M K$_2$HPO$_4$ and submitted to a new centrifugation at 21,000 $\times g$ 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 [36,37]. 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 $\pm$ standard deviation. 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* 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 ($\Delta\Psi$m)

We first observed that 3 HTA significantly decreases $\Delta\Psi$m in a dose dependent manner and that this effect is enhanced when mitochondrion was challenged by Ca^{2+} (Fig. 1A). Furthermore, 3 HTA-induced mitochondrial depolarization was prevented by RR, that blocks Ca^{2+} entrance into the mitochondria, and by CsA plus ADP, classical inhibitors of mPTP (Fig. 1B). Fig. 1C shows that 3 HPA similarly decreased $\Delta\Psi$m after Ca^{2+} addition, that was prevented by CsA plus ADP. These data indicate that 3 HTA and 3 HPA elicit mPTP and that this effect is dependent on the presence of Ca^{2+} in the mitochondrial preparations.

3.2. LCHFA provoke mitochondrial swelling

Then, we investigated the influence of the LCFA on mitochondrial swelling. We verified that these fatty acids induced a significant but differential swelling in Ca^{2+}-loaded mitochondria pending on their chain length (Fig. 2A–D). So, mitochondrial swelling was increased 6-fold by 3 HPA (Fig. 2B), 3.5-fold by 3 HTA (Fig. 2A) and 2-fold by 3 HDA (Fig. 2C). Furthermore, the effects provoked by 3 HTA was avoided by RR and by CsA plus ADP, implying a role for Ca^{2+}, as well as the involvement of mPTP opening, in 3 HTA-induced mitochondrial swelling (Fig. 3).

3.3. Mitochondrial Ca²⁺ retention capacity is reduced by 3 HTA

We evaluated the effects of 3 HTA on mitochondrial Ca^{2+} retention capacity, since one of the critical functions of the mitochondria is to
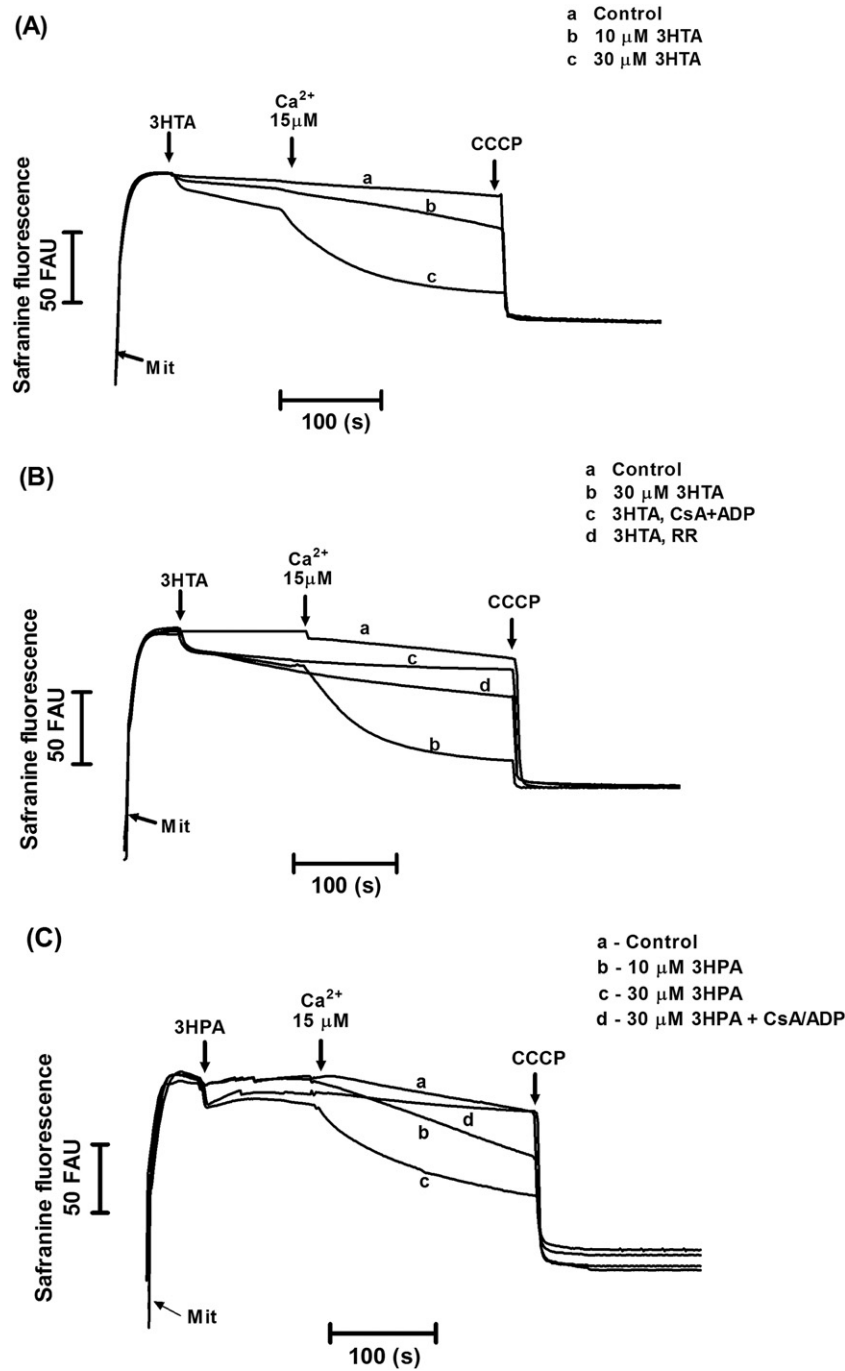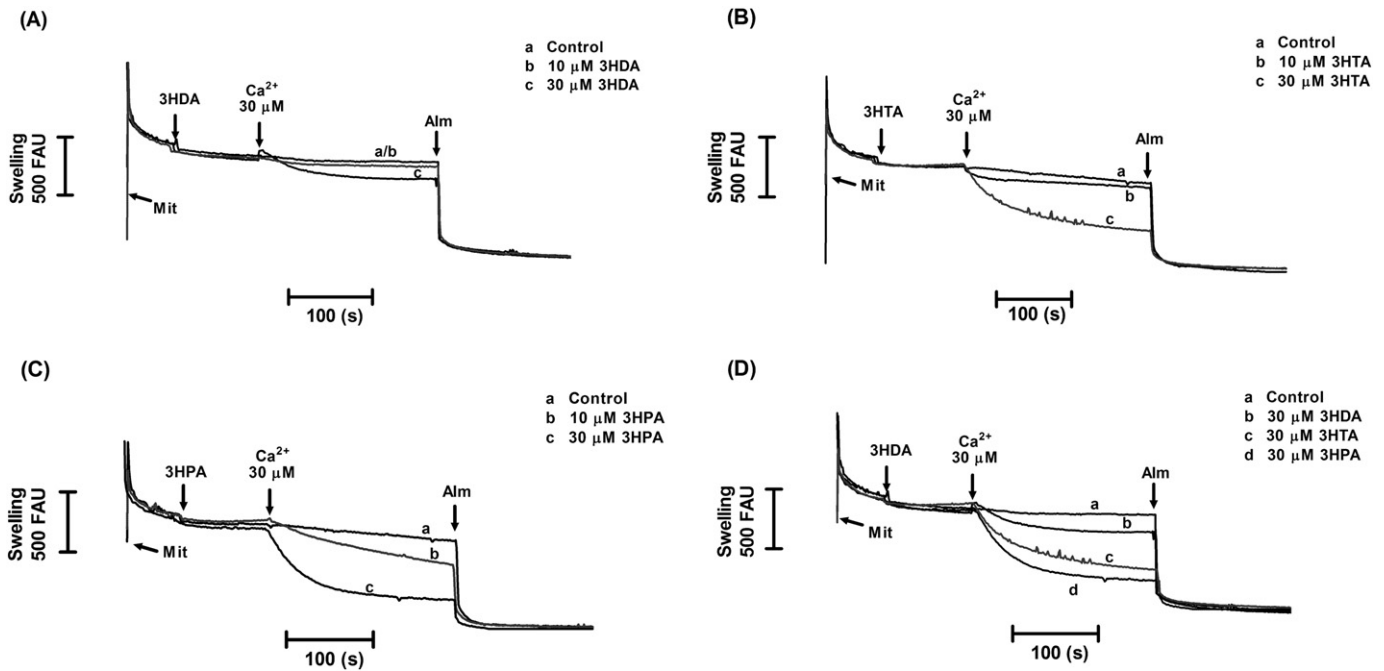
Fig. 1. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) and 3-hydroxypalmitic acid (3 HPA) on mitochondrial membrane potential in the presence of Ca^{2+}. (A) 3 HTA (10–30 μM, lines b–c) was added 50 s after the beginning of the assay to the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported by glutamate/malate) and followed by addition of 15 μM Ca^{2+} 100 s later, as indicated. (B) 30 μM 3 HTA (lines b–d) was added 50 s after the beginning of the assay to the reaction media and followed by addition of 15 μM Ca^{2+} 100 s later, 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. (C) 3 HPA (10–30 μM, lines b–d) were added 50 s after the beginning of the assay to the reaction media and followed by addition of 15 μM Ca^{2+} 100 s later, as indicated. CsA (1 μM) plus ADP (300 μM) (line c) were added in the beginning of the assay. Controls (line a) were performed in the absence of 3 HTA or 3 HPA with addition of 15 μM Ca^{2+} 150 s after the beginning of the assay. Fluorescence changes between 150 and 250 s were: (A) 6.79 ± 0.76 (control), 19.5 ± 3.25 (10 μM 3 HTA) and 52.8 ± 8.06 (30 μM 3 HTA), [$F_{(2,6)}$ = 66.92, P < 0.001] compared between the groups; (B) 7.39 ± 0.34 (control), 49.7 ± 17.2 (30 μM 3 HTA), 4.63 ± 0.52 (30 μM 3 HTA + CsA/ADP) and 10.4 ± 0.89 (30 μM 3 HTA + RR), [$F_{(3,8)}$ = 18.3, P < 0.001] compared between the groups; (C) 6.64 ± 0.72 (control), 16.3 ± 4.68 (10 μM 3 HPA), 26.1 ± 3.68 (30 μM 3 HPA) and 5.12 ± 1.64 (30 μM 3 HPA + CsA/ADP), [$F_{(3,8)}$ =29.41, P < 0.001] compared between the groups. 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).

control cellular Ca^{2+} homeostasis [38]. Furthermore, mPTP induction may lead to Ca^{2+} release from mitochondria. It was found that 3 HTA reduced Ca^{2+} retention capacity in mitochondria supported by glutamate plus malate whose effect was prevented by CsA plus ADP, indicating the involvement of mPTP (Fig. 4).

3.4. 3 HTA reduces NAD(P)H mitochondrial matrix content

The effect of 3 HTA on NAD(P)H matrix content was also evaluated (Fig. 5). We observed that 3 HTA induced a decrease of NAD(P)H levels in the presence of Ca^{2+}. The data suggest that the reduced equivalents

Fig. 2. *In vitro* effects of long-chain 3-hydroxy fatty acids (LCHFA) on mitochondrial swelling in the presence of Ca^{2+}. Increasing concentrations (10–30 μM, lines b–c) of (A) 3-hydroxydodecanoic acid (3 HDA), (B) 3-hydroxytetradecanoic acid (3 HTA) or (C) 3-hydroxypalmitic acid (3 HPA) were added 50 s 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 addition of 30 μM Ca^{2+} 100 s later, as indicated. A comparison of 30 μM 3 HDA, 3 HTA and 3 HPA is depicted in panel (D). Controls (line a) were performed in the absence of 3 HTA with addition of 30 μM Ca^{2+} 150 s after the beginning of the assay. Fluorescence changes between 150 and 250 s were: (A) 64 ± 19.5 (control), 103 ± 6.09 (10 μM 3 HDA) and 196 ± 19.9 (30 μM 3 HDA), [$F_{(2,6)}$ = 51.36, P < 0.001] compared between the groups; (B) 64 ± 19.5 (control), 133 ± 28 (10 μM 3 HTA) and 286 ± 72 (30 μM 3 HTA), [$F_{(2,6)}$ = 18.36, P < 0.01] compared between the groups; (C) 64 ± 19.5 (control), 194 ± 85.4 (10 μM 3 HPA) and 435 ± 11.1 (30 μM 3 HPA), [$F_{(2,6)}$ = 40.85, P < 0.001] compared between the groups; (D) 64 ± 19.5 (control), 196 ± 19.9 (30 μM 3 HDA), 286 ± 72 (30 μM 3 HTA) and 435 ± 11.1 (30 μM 3 HPA), [$F_{(3,8)}$ = 47.91, P < 0.001] compared between the groups. Alamethicin (Alm, 40 μg/mg of protein) was added at the end of the measurements. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU).

were oxidized or lost from the mitochondrial matrix (Fig. 5A). Furthermore CsA plus ADP and RR prevented the decrease of NAD(P)H content induced by 3 HTA (Fig. 5B).

3.5. 3 HTA increases 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 can be induced 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 when Ca^{2+} was added to the mitochondrial preparations. It can be also observed in the figure that CsA plus ADP and RR completely prevented 3 HTA-induced H_2O_2 increase, highlighting the synergistic role of 3 HTA and Ca^{2+} inducing mPTP.

3.6. 3 HTA induces cytochrome c release

Fig. 7 shows a significant decrease of cytochrome *c* immunocontent in mitochondria treated with 3 HTA and Ca^{2+}, suggesting cytochrome *c* release. Furthermore, this effect was prevented by CsA plus ADP and by
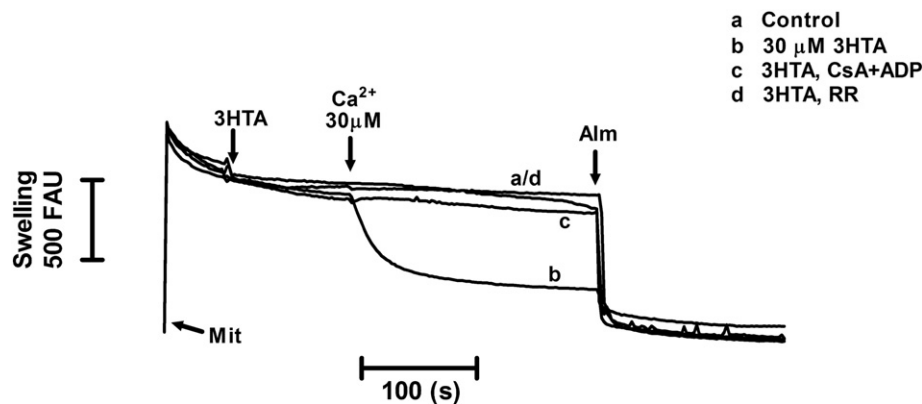
Fig. 3. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial swelling in the presence of Ca^{2+}. 30 μM 3 HTA (lines b–d) was added 50 s after the beginning of the assay in the reaction media containing mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported by glutamate/malate) and followed by addition of 30 μM Ca^{2+} 100 s later, as indicated. In some experiments cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line c) or ruthenium red (RR, 1 μM, line d) was added in the beginning of the assay. Controls (line a) were performed in the absence of 3 HTA with addition of 30 μM Ca^{2+} 150 s after the beginning of the assay. Fluorescence changes between 150 and 250 s were: (A) 90.7 ± 24.3 (control), 328 ± 103 (30 μM 3 HTA), 47.3 ± 17.6 (30 μM 3 HTA + CsA/ADP) and 40.7 ± 34.1 (30 μM 3 HTA + RR), [$F_{(3,8)}$ = 17.56, P < 0.001] compared between the groups. Alamethicin (Alm, 40 μg/mg of protein) was added at the end of the measurements. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU).
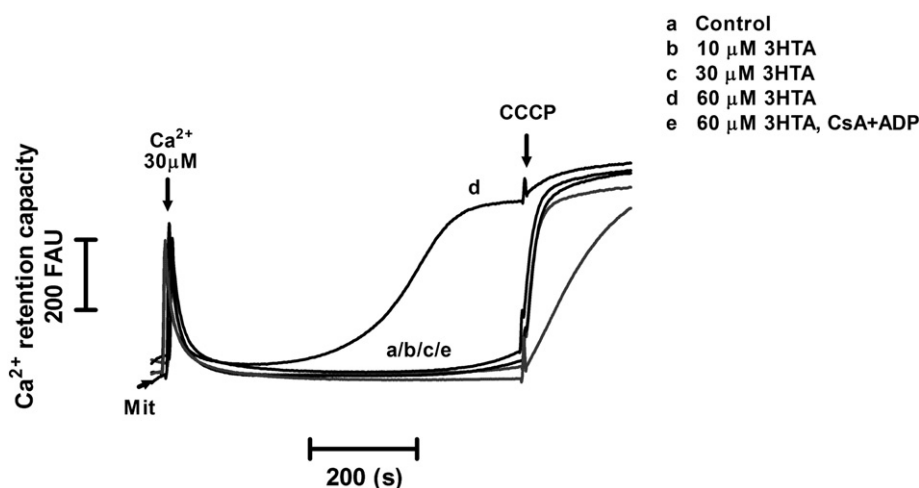
Fig. 4. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial Ca^{2+} retention capacity. Ca^{2+} (30 μM) was added 100 s after the beginning of the assay in the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported by glutamate/malate), 30 μM ADP and 3 HTA (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 addition of 30 μM Ca^{2+} 100 s after the beginning of the assay. Fluorescence changes between 250 and 500 s were: (A) 15.8 ± 4.38 (control), 15.8 ± 3.5 (10 μM 3 HTA), 15.1 ± 2.6 (30 μM 3 HTA), 188 ± 138 (60 μM 3 HTA) and 21 ± 2.01 (60 μM 3 HTA + CsA/ADP), [$F_{(4,10)}$ = 4.593, P < 0.05] compared between the groups. 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).

RR, supporting the involvement of mPTP on 3 HTA-induced cytochrome *c* reduction. It can be also observed in the figure that the supplementation of CsA plus ADP and RR to the mitochondrial preparations resulted in a higher level of cytochrome *c*, as compared to controls. This may have occurred due to the induction of mPTP caused by Ca^{2+}. Alm was used as a positive control once it induces mitochondrial pore formation.

3.7. LCHFA decrease ATP levels

Finally, we tested whether LCHFA could alter ATP production. We verified that 3 HDA, 3 HTA and 3 HPA significantly decreased ATP content especially when incubated in the presence of Ca^{2+} (Fig. 8), probably as a consequence of disruption of mitochondrial energy production. It can be also seen in the figure that the degree of this effect was dependent on the fatty acid carbon chain length, so that the longer the carbon chain the greater the effect observed.

4. Discussion

In the present study we showed that the LCHFA accumulated in LCHAD and MTP deficiencies change various parameters of mitochondrial bioenergetics indicative of mPTP opening in Ca^{2+}-loaded mitochondria from cerebral cortex of adolescent rats. We first demonstrated that 3 HTA dissipates mitochondrial membrane potential (ΔΨm) and that this effect was much higher after Ca^{2+} addition. Furthermore, ruthenium red (RR), a potent inhibitor of the mitochondrial Ca^{2+} uniporter [39], prevented ΔΨm dissipation, supporting a role for Ca^{2+} in this effect. In addition, 3 HTA-induced ΔΨm decrease was probably caused by mPTP since it was accompanied by swelling and fully prevented by CsA plus ADP and RR. It is emphasized that CsA is an effective peptide inhibitor of mPTP by binding to cyclophilin D, a mitochondrial matrix protein that interacts with the adenine nucleotide translocator modulating the pore opening [40–43].

Since one of the most important functions of the mitochondria is to control Ca^{2+} intracellular concentrations and dysregulation of Ca^{2+} homeostasis is associated with disturbance of energy and redox homeostasis [44–50], we tested whether 3 HTA could affect mitochondrial Ca^{2+} retention capacity. We observed that 3 HTA compromised the mitochondrial Ca^{2+} retention capacity and that this effect was prevented by CsA plus ADP and RR, reinforcing a synergistic role for Ca^{2+} and 3 HTA inducing mPTP. Taken together, it is conceivable that mPTP

opening could allow Ca^{2+} release from the matrix after reaching a threshold overcoming mitochondrial Ca^{2+} retention capacity, resulting in a nonselective inner membrane permeabilization [51–53].

3 HTA also diminished matrix NAD(P)H content after Ca^{2+} loading that was avoided 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 mPTP. Alternatively, NADH consumption resulting from activation of the electron transport flow that enhances its oxidation, may also underlie the reduction of NAD(P)H observed in our investigation [54–56].

As regards the mechanisms by which LCHFA cause mPTP opening in Ca^{2+}-loaded mitochondria, this remains an open question. However, it should be considered that induction of mPTP can be provoked by uncouplers of oxidative phosphorylation, as well as by oxidation of membrane protein thiol groups leading to nonselective permeabilization [51, 56–61]. Thus, considering that LCFA and particularly LCHFA were shown to uncouple oxidative phosphorylation and to provoke oxidative stress and protein oxidation [12,62,63,13,14,64,65], it is tempting to speculate that these pathomechanisms may be involved in mPTP induction by these fatty acids. On the other hand, the induction of mitochondrial H_2O_2 production caused by the synergistic effect of 3 HTA and Ca^{2+} was prevented by CsA plus ADP and by RR, indicating that H_2O_2 increase was a consequence rather than a cause of mPTP opening, as previously found in other situations [66–69].

On the other hand, it is of note that saturated LCFA and their α,ω-dioic acids can also induce nonspecific permeability of the inner membrane in liver mitochondria loaded with Ca^{2+}, although by other mechanisms that are insensitive to CsA [16,70]. Our present data allied to previous findings indicate that the LCHFA and the non-hydroxylated LCFA induce nonselective permeabilization by distinct mechanisms.

3 HTA also reduced mitochondrial cytochrome *c* immunocontent in Ca^{2+}-loaded mitochondria, probably due to increase of nonselective inner membrane permeabilization (mPTP) caused by this fatty acid plus Ca^{2+}. The release of cytochrome *c* from the mitochondrial intermembrane space into the cytosol provoked by mPTP typically accompanies the osmotic swelling and the physical rupture of the mitochondrial outer membrane [71,72]. Our observations that cytochrome release *c* was prevented by CsA plus ATP or by RR support this hypothesis. In addition, since the release of cytochrome *c* from mitochondria into the cytosol precede the apoptosis induction by forming the
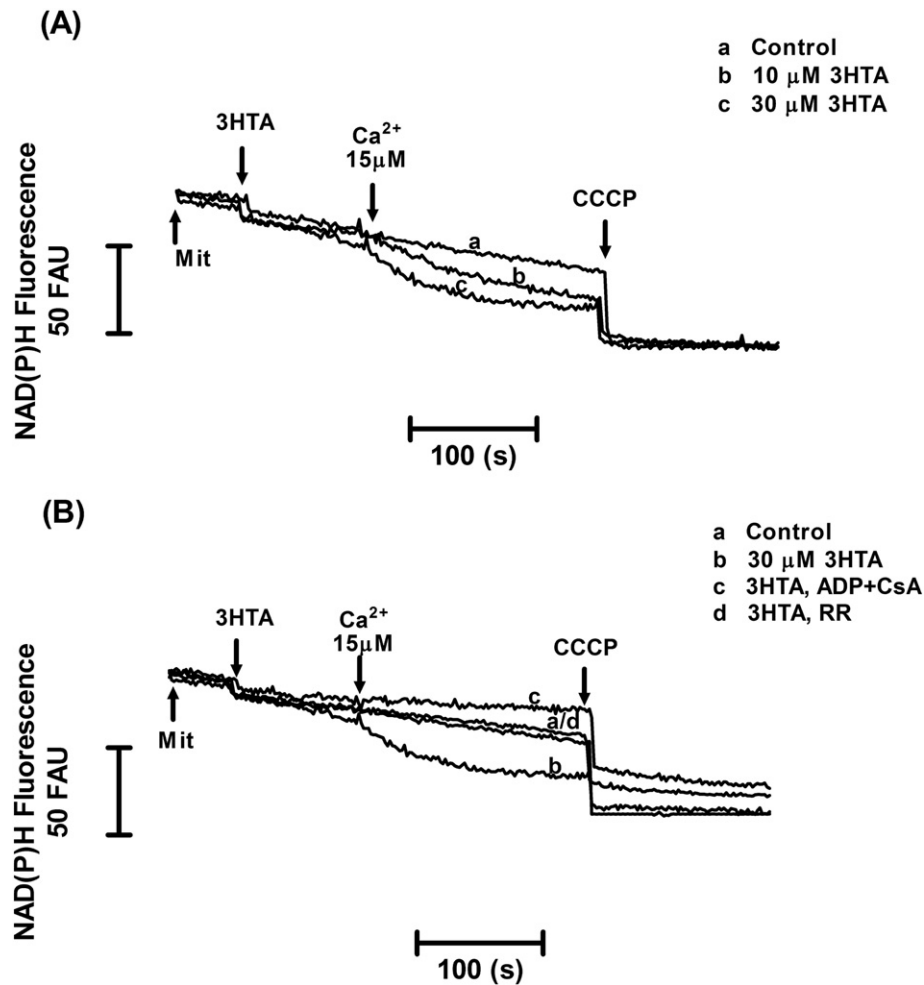
Fig. 5. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial NADH and NADPH content in the presence of Ca^{2+}. (A) 3 HTA (10 μM–30 μM, lines b–c) was added 50 s after the beginning of the assay to the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported by glutamate/malate) and followed by addition of 15 μM Ca^{2+} 100 s later, as indicated. (B) 30 μM 3 HTA (lines b–d) was added 50 s after the beginning of the assay to the reaction media and followed by addition of 15 μM Ca^{2+} 100 s later. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line c) or ruthenium red (RR, 1 μM, line d) was added in the beginning of the assay. Controls (line a) were performed in the absence of 3 HTA with addition of 15 μM Ca^{2+} 150 s after the beginning the assay. Fluorescence changes between 150 and 250 s were: (A) 8.09 ± 1.22 (control), 12.4 ± 1.06 (10 μM 3 HTA) and 17.8 ± 1.63 (30 μM 3 HTA), $[F_{(2,6)} = 40.28, P < 0.001]$ compared between the groups; (B) 8.09 ± 1.22 (control), 17.8 ± 1.63 (30 μM 3 HTA), 5.27 ± 2.82 (30 μM 3 HTA + CsA/ADP) and 4.27 ± 0.59 (30 μM 3 HTA + RR), $[F_{(3,8)} = 36.71, P < 0.001]$ compared between the groups. CCCP (3 μM) was added in the end of the assays, as indicated. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU).

apoptosome complex with Apaf-1 and procaspase-9 and initiating the caspase cascade [73–75], it is conceivable that 3 HTA may potentially 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 other LCHFA, reflecting a deleterious consequence to mitochondrial functions. ATP decrease may have occurred due to the uncoupling effect of these fatty acids causing impairment in the maintenance of ΔΨm necessary to ATP biosynthesis [76], 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 ΔΨm and/or loss of adenine nucleotides to the external media through mPTP formation [73,77].

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 was dependent on the size of the carbon chain.

At the present it is difficult to establish the pathophysiological significance of our data since the brain levels of LCHFA in patients affected by LCHAD and MTP deficiencies are still unknown. However, we showed

here that the LCHFA that mostly accumulate in these disorders, particularly 3 HPA and 3 HTA, reduced the mitochondrial membrane potential and provoked swelling in the presence of calcium that was prevented by CsA, ADP and RR at similar or even lower concentrations (10 μM) than those found in plasma of the affected patients, especially during crises of metabolic decompensation that are accompanied by encephalopathy with coma [78,79]. It is emphasized that these crises generally follow infections and accelerated catabolism that lead to increased tissue levels of these fatty acids [79]. These data strongly indicate that the LCHFA induce mPTP, a deleterious condition that causes disruption of the mitochondrial homeostasis. Therefore, it is presumed that our results may be of pathophysiological relevance, especially during metabolic crises.

In a previous study we showed that the LCHFA accumulating in LCHAD and MTP deficiencies cause uncoupling of oxidative phosphorylation by altering the respiratory parameters measured by oxymetry and besides elicited mPTP in purified mitochondrial preparations from cardiac muscle of adolescent rats [13]. In the present investigation we demonstrated that besides inducing mPTP, these fatty acids substantially decreased ATP production and provoked significant cytochrome release in brain, that are indicative of a mitochondrial bioenergetics

(A)

a Control
b 60 μM 3HTA
c 3HTA, CsA+ADP
d 3HTA, RR

(B)
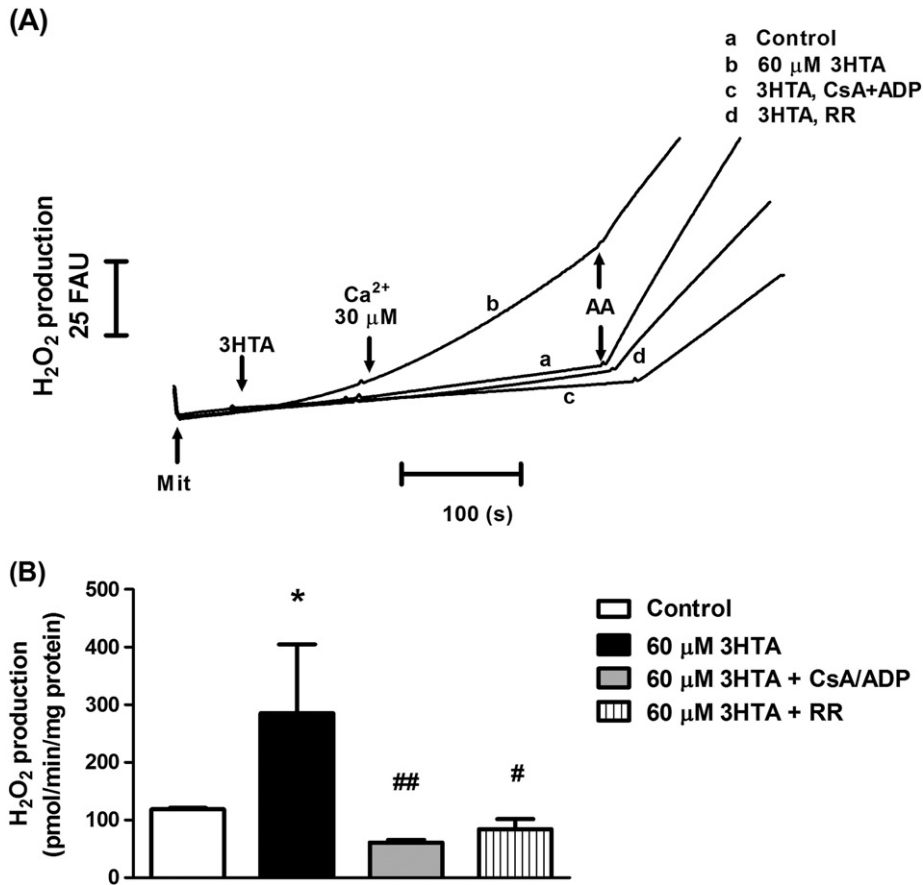
Fig. 6. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial hydrogen peroxide (H$_2$O$_2$) production in the presence of Ca^{2+}. (A) 60 μM 3 HTA (line b) was added 50 s after the beginning of the assay in the reaction media containing the mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported by glutamate/malate) and followed by addition of 30 μM Ca^{2+} 100 s later, as indicated. Cyclosporin A (CsA, 1 μM) plus ADP (300 μM) (line c) or ruthenium red (RR, 1 μM, line d) was added in the beginning of the assay. Controls (line a) were performed in the absence of 3 HTA with addition of 30 μM Ca^{2+} 150 s after the beginning of the assay. Antimycin A (AA, 0.1 mg/mL) was added at the end of the measurements. Traces are representative of three independent experiments (animals) and were expressed as fluorescence arbitrary units (FAU). (B) Quantitative H$_2$O$_2$ production representing means ± standard deviation as a bar graph for three independent experiments (animals) and expressed as pmol/min/mg protein. *P < 0.05 compared to control; #P < 0.05, ##P < 0.01 compared to 60 μM 3 HTA (Tukey's multiple comparison test).
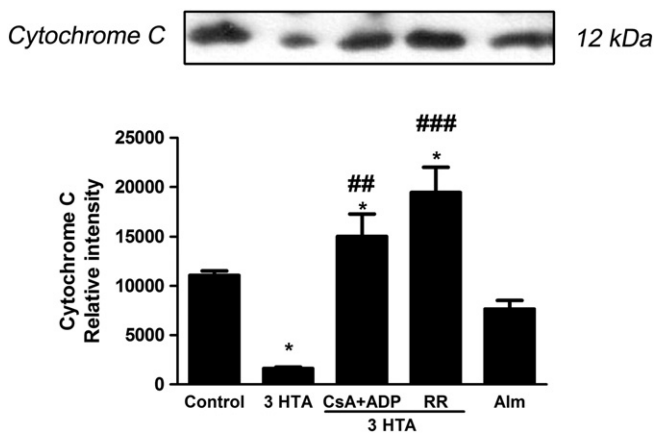
Fig. 7. *In vitro* effects of 3-hydroxytetradecanoic acid (3 HTA) on mitochondrial cytochrome *c* immunocontent. 30 μM 3 HTA was added 50 s after the beginning of the assay in the reaction media containing the mitochondrial preparations (0.5 mg protein·mL^{-1} supported by glutamate/malate) and followed by addition of 30 μM Ca^{2+} 100 s later. Controls were performed in the absence of 3 HTA with addition of 30 μM Ca^{2+} 150 s after the beginning of the assay. In some experiments cyclosporin A (CsA, 1 μM) plus ADP (300 μM) or ruthenium red (RR, 1 μM) was 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 (animals) and were expressed as relative intensity. *P < 0.05 **P < 0.01, ***P < 0.001 compared to control (Tukey's multiple comparison test).
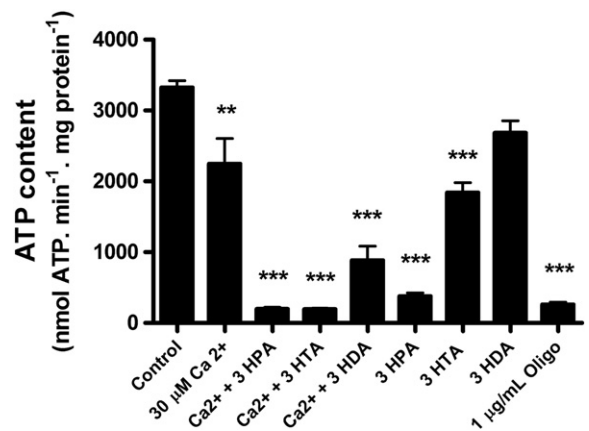
Fig. 8. *In vitro* effects of long-chain 3-hydroxy fatty acids (LCHFA) on ATP content in mitochondrial preparations (Mit, 0.5 mg protein·mL^{-1} supported glutamate/malate). 30 μM 3-hydroxydodecanoic acid (3 HDA), 3-hydroxytetradecanoic acid (3 HTA) and 3-hydroxypalmitic acid (3 HPA) were added in the beginning of the assay. In some experiments, 30 μM Ca^{2+} was added 90 s later. Controls were performed in the absence of LCHFA with addition of 30 μM Ca^{2+} 150 s 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 were 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).

collapse. Therefore, we may presume that a disturbed mitochondrial energy homeostasis caused by the LCHFA in heart and brain may represent a pathomechanism involved in the clinical manifestations occurring in the affected patients.

5. Conclusion

In conclusion, our study provides for the first time evidence that LCHFA act synergistically with Ca^{2+} inducing mPTP and disrupting mitochondrial energy homeostasis in the brain tissue. We showed a permanent pore opening that strongly compromised mitochondrial functions as maintenance of $\Delta\Psi m$ and NAD(P)H pool, as well as ATP content and Ca^{2+} homeostasis, leading to cytochrome *c* release and possibly culminating in apoptotic (or necrotic) cell death [73]. It is proposed that disruption of energy homeostasis may be associated with the long-term neurologic manifestations presented by patients affected by LCHAD and MTP deficiencies. In this context, there is a growing body of evidence that mPTP opening contributes to tissue injury in a variety of diseases and is closely associated to neurodegeneration [80–82].

Acknowledgments

References

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

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

[3] C. Roe, J. Ding, Mitochondrial fatty acids oxidation disorders, in: S.C.R., B.A.L., S.W.S., V.D. (Eds.), The Metabolic and Molecular Bases of Inherited Disease, McGraw-Hill, New York, 2001, pp. 1909–1963.

[4] S. Ushikubo, T. Aoyama, T. Kamijo, R.J. Wanders, P. Rinaldo, J. Vockley, T. Hashimoto, Molecular characterization of mitochondrial trifunctional protein deficiency: formation of the enzyme complex is important for stabilization of both alpha- and beta-subunits, Am. J. Hum. Genet. 58 (1996) 979–988.

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

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

[7] 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.

[8] U. Spiekerkoetter, Z. Khuchua, Z. Yue, M.J. Bennett, A.W. Strauss, General mitochondrial 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.

[9] 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.

[10] 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 (2013) S31–S39 (Suppl.).

[11] M. Wakabayashi, Y. Kamijo, T. Nakajima, N. Tanaka, E. Sugiyama, T. Yangyang, T. Kimura, T. Aoyama, Fatty acid accumulation and resulting PPARalpha activation in fibroblasts due to trifunctional protein deficiency, PPAR Res. 2012 (2012) 371691.

[12] A.M. Tonin, M. Grings, E.N. Busanello, A.P. Moura, G.C. Ferreira, C.M. Viegas, C.G. Fernandes, P.F. Schuck, M. Wajner, Long-chain 3-hydroxy fatty acids accumulating in LCHAD and MTP deficiencies induce oxidative stress in rat brain, Neurochem. Int. 56 (2010) 930–936.

[13] 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 dehydrogenase and mitochondrial trifunctional protein deficiencies uncouple oxidative phosphorylation in heart mitochondria, J. Bioenerg. Biomembr. 45 (2013) 47–57.

[14] 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.

[15] 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 dehydrogenase deficiency with the G1528C mutation, Neuromuscul. Disord. 6 (1996) 327–337.

[16] A. Sultan, P.M. Sokolove, Palmitic acid opens a novel cyclosporin A-insensitive pore in the inner mitochondrial membrane, Arch. Biochem. Biophys. 386 (2001) 37–51.

[17] N. Auestad, R.A. Korsak, J.W. Morrow, J. Edmond, Fatty acid oxidation and ketogenesis by astrocytes in primary culture, J. Neurochem. 56 (1991) 1376–1386.

[18] J. Edmond, T.A. Higa, R.A. Korsak, E.A. Bergner, W.N. Lee, Fatty acid transport and utilization for the developing brain, J. Neurochem. 70 (1998) 1227–1234.

[19] J. Edmond, R.A. Korsak, J.W. Morrow, G. Torok-Both, D.H. Catlin, Dietary cholesterol and the origin of cholesterol in the brain of developing rats, J. Nutr. 121 (1991) 1323–1330.

[20] D. Ebert, R.G. Haller, M.E. Walton, Energy contribution of octanoate to intact rat brain metabolism measured by ^{13}C nuclear magnetic resonance spectroscopy, J. Neurosci. 23 (2003) 5928–5935.

[21] J.A. Hamilton, Transport of fatty acids across membranes by the diffusion mechanism, Prostaglandins Leukot. Essent. Fat. Acids 60 (1999) 291–297.

[22] R. Spector, Fatty acid transport through the blood–brain barrier, J. Neurochem. 50 (1988) 639–643.

[23] W.M. Williams, M.C. Chang, T. Hayakawa, E. Grange, S.I. Rapoport, In vivo incorporation from plasma of radiolabeled palmitate and arachidonate into rat brain microvessels, Microvasc. Res. 53 (1997) 163–166.

[24] R.W. Mitchell, G.M. Hatch, Fatty acid transport into the brain: of fatty acid fables and lipid tails, Prostaglandins Leukot. Essent. Fat. Acids 85 (2011) 293–302.

[25] Q.R. Smith, H. Nagura, Fatty acid uptake and incorporation in brain: studies with the perfusion model, J. Mol. Neurosci. 16 (2001) 167–172 (discussion 215–121).

[26] M. Pallebage-Gamarallage, V. Lam, R. Takechi, S. Galloway, K. Clark, J. Mamo, Restoration of dietary-fat induced blood–brain barrier dysfunction by anti-inflammatory lipid-modulating agents, Lipids Health Dis. 11 (2012) 117.

[27] P.A. Fraser, The role of free radical generation in increasing cerebrovascular permeability, Free Radic. Biol. Med. 51 (2011) 967–977.

[28] B. Davletov, C. Montecucco, Lipid function at synapses, Curr. Opin. Neurobiol. 20 (2010) 543–549.

[29] R. Rosenthal, F. Hamud, G. Fiskum, P. Varghese, S. Sharpe, Cerebral ischemia and reperfusion: prevention of brain mitochondrial injury by lidoflazine, J. Cereb. Blood Flow Metab. 7 (1987) 752–758.

[30] 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.

[31] 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.

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

[33] A.J. Kowaltowski, R.G. Cosso, C.B. Campos, G. Fiskum, Effect of Bcl-2 overexpression on mitochondrial structure and function, J. Biol. Chem. 277 (2002) 42802–42807.

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

[35] J.G. Mohanty, J.S. Jaffe, E.S. Schulman, D.G. Raible, A highly sensitive fluorescent micro-assay of H_2O_2 release from activated human leukocytes using a dihydroxyphenoxazine derivative, J. Immunol. Methods 202 (1997) 133–141.

[36] J.J. Lemasters, C.R. Hackenbrock, Continuous measurement and rapid kinetics of ATP synthesis in rat liver mitochondria, mitoplasts and inner membrane vesicles determined by firefly-luciferase luminescence, Eur. J. Biochem. 67 (1976) 1–10.

[37] M.A. Maioli, D.E. Lemos, M. Guelfi, H.C. Medeiros, F. Riet-Correa, R.M. Medeiros, J.M. Barbosa-Filho, F.E. Mingatto, Mechanism for the uncoupling of oxidative phosphorylation by juliprosopine on rat brain mitochondria, Toxicon 60 (2012) 1355–1362.

[38] 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.

[39] L.E. Moore, Effect of temperature and calcium ions on rate constants of myelinated nerve, Am. J. Physiol. 221 (1971) 131–137.

[40] C. Yarana, J. Sripetchwandee, J. Sanit, S. Chattipakorn, N. Chattipakorn, Calcium-induced cardiac mitochondrial dysfunction is predominantly mediated by cyclosporine A-dependent mitochondrial permeability transition pore, Arch. Med. Res. 43 (2012) 333–338.

[41] J. Pottecher, M. Guillot, E. Belaidi, A.L. Charles, A. Lejay, A. Gharib, P. Diemunsch, B. Geny, Cyclosporine A normalizes mitochondrial coupling, reactive oxygen species production, and inflammation and partially restores skeletal muscle maximal oxidative capacity in experimental aortic cross-clamping, J. Vasc. Surg. 57 (2013) (1100-1108 e1102).

[42] 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.

[43] A. Tanveer, S. Virji, L. Andreeva, N.F. Totty, J.J. Hsuan, J.M. Ward, M. Crompton, Involvement of cyclophilin D in the activation of a mitochondrial pore by $Ca2+$ and oxidant stress, Eur. J. Biochem. 238 (1996) 166–172.

[44] S. Gandhi, A. Wood-Kaczmar, Z. Yao, H. Plun-Favreau, E. Deas, K. Klupsch, J. Downward, D.S. Latchman, S.J. Tabrizi, N.W. Wood, M.R. Duchen, A.Y. Abramov, PINK1-associated Parkinson's disease is caused by neuronal vulnerability to calcium-induced cell death, Mol. Cell 33 (2009) 627–638.

[45] M.K. Jaiswal, W.D. Zech, M. Goos, C. Leutbecher, A. Ferri, A. Zippelius, M.T. Carri, R. Nau, B.U. Keller, Impairment of mitochondrial calcium handling in a mtSOD1 cell culture model of motoneuron disease, BMC Neurosci. 10 (2009) 64.

[46] L.J. Martin, B. Gertz, Y. Pan, A.C. Price, J.D. Molkentin, Q. Chang, The mitochondrial permeability transition pore in motor neurons: involvement in the pathobiology of ALS mice, Exp. Neurol. 218 (2009) 333–346.

[47] R.A. Quintanilla, G.V. Johnson, Role of mitochondrial dysfunction in the pathogenesis of Huntington's disease, Brain Res. Bull. 80 (2009) 242–247.

[48] T.R. Rosenstock, A.C. Carvalho, A. Jurkiewicz, R. Frussa-Filho, S.S. Smaili, Mitochondrial calcium, oxidative stress and apoptosis in a neurodegenerative disease model induced by 3-nitropropionic acid, J. Neurochem. 88 (2004) 1220–1228.

[49] R.F. Castilho, O. Hansson, M.W. Ward, S.L. Budd, D.G. Nicholls, Mitochondrial control of acute glutamate excitotoxicity in cultured cerebellar granule cells, J. Neurosci. 18 (1998) 10277–10286.

[50] A.K. Stout, H.M. Raphael, B.I. Kanterewicz, E. Klann, I.J. Reynolds, Glutamate-induced neuron death requires mitochondrial calcium uptake, Nat. Neurosci. 1 (1998) 366–373.

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

[52] 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.

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

[54] 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.

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

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

[57] R.F. Castilho, A.J. Kowaltowski, A.R. Meinicke, E.J. Bechara, A.E. Vercesi, Permeabilization of the inner mitochondrial membrane by Ca^{2+} ions is stimulated by t-butyl hydroperoxide and mediated by reactive oxygen species generated by mitochondria, Free Radic Biol Med. 18 (1995) 479–486.

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

[59] A.J. Kowaltowski, A.E. Vercesi, R.F. Castilho, Mitochondrial membrane protein thiol reactivity with N-ethylmaleimide or mersalyl is modified by Ca2+: correlation with mitochondrial permeability transition, Biochim. Biophys. Acta 1318 (1997) 395–402.

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

[61] A.J. Kowaltowski, R.F. Castilho, A.E. Vercesi, Opening of the mitochondrial permeability transition pore by uncoupling or inorganic phosphate in the presence of Ca2+ is dependent on mitochondrial-generated reactive oxygen species, FEBS Lett. 378 (1996) 150–152.

[62] P.F. Schuck, G.C. Ferreira, A.P. Moura, E.N. Busanello, A.M. Tonin, C.S. Dutra-Filho, M. Wajner, Medium-chain fatty acids accumulating in MCAD deficiency elicit lipid and protein oxidative damage and decrease non-enzymatic antioxidant defenses in rat brain, Neurochem. Int. 54 (2009) 519–525.

[63] G. Leipnitz, A. Amaral, A. Zanatta, B. Seminotti, C. Fernandes, L. Knebel, C. Vargas, M. Wajner, Neurochemical evidence that phytanic acid induces oxidative damage and reduces the antioxidant defenses in cerebellum and cerebral cortex of rats, Life Sci. 87 (2010) 275–280.

[64] V.N. Samartsev, E.I. Marchik, L.V. Shamagulova, Free fatty acids as inducers and regulators of uncoupling of oxidative phosphorylation in liver mitochondria with participation of ADP/ATP- and aspartate/glutamate-antiporter, Biochem. Biokhim. 76 (2011) 217–224.

[65] P.F. Schuck, C. Ferreira Gda, A.M. Tonin, C.M. Viegas, E.N. Busanello, A.P. Moura, A. Zanatta, F. Klamt, M. Wajner, Evidence that the major metabolites accumulating in medium-chain acyl-CoA dehydrogenase deficiency disturb mitochondrial energy homeostasis in rat brain, Brain Res. 1296 (2009) 117–126.

[66] C. Penna, M.G. Perrelli, P. Pagliaro, Mitochondrial pathways, permeability transition pore, and redox signaling in cardioprotection: therapeutic implications, Antioxid. Redox Signal. 18 (2013) 556–599.

[67] D.B. Zorov, M. Juhaszova, S.J. Sollott, Mitochondrial ROS-induced ROS release: an update and review, Biochim. Biophys. Acta 1757 (2006) 509–517.

[68] M.A. Aon, S. Cortassa, C. Maack, B. O'Rourke, Sequential opening of mitochondrial ion channels as a function of glutathione redox thiol status, J. Biol. Chem. 282 (2007) 21889–21900.

[69] D.B. Zorov, C.R. Filburn, L.O. Klotz, J.L. Zweier, S.J. Sollott, Reactive oxygen species (ROS)-induced ROS release: a new phenomenon accompanying induction of the mitochondrial permeability transition in cardiac myocytes, J. Exp. Med. 192 (2000) 1001–1014.

[70] M.V. Dubinin, S.I. Adakeeva, V.N. Samartsev, Long-chain alpha, omega-dioic acids as inducers of cyclosporin A-insensitive nonspecific permeability of the inner membrane of liver mitochondria loaded with calcium or strontium ions, Biochem. Biokhim. 78 (2013) 412–417.

[71] M. Crompton, Mitochondrial intermembrane junctional complexes and their role in cell death, J. Physiol. 529 (Pt 1) (2000) 11–21.

[72] P.X. Petit, M. Goubern, P. Diolez, S.A. Susin, N. Zamzami, G. Kroemer, Disruption of the outer mitochondrial membrane as a result of large amplitude swelling: the impact of irreversible permeability transition, FEBS Lett. 426 (1998) 111–116.

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

[74] D.R. Green, J.C. Reed, Mitochondria and apoptosis, Science 281 (1998) 1309–1312.

[75] A. Andreyev, G. Fiskum, Calcium induced release of mitochondrial cytochrome c by different mechanisms selective for brain versus liver, Cell Death Differ. 6 (1999) 825–832.

[76] M.D. Brand, D.G. Nicholls, Assessing mitochondrial dysfunction in cells, Biochem. J. 435 (2011) 297–312.

[77] P. Bernardi, A. Rasola, Calcium and cell death: the mitochondrial connection, Subcell. Biochem. 45 (2007) 481–506.

[78] 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.

[79] 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.

[80] A.N. Murphy, G. Fiskum, M.F. Beal, Mitochondria in neurodegeneration: bioenergetic function in cell life and death, J. Cereb. Blood Flow Metab. 19 (1999) 231–245.

[81] H. Friberg, T. Wieloch, Mitochondrial permeability transition in acute neurodegeneration, Biochimie 84 (2002) 241–250.

[82] 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.