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Pharmacological inhibition of carnitine palmitoyltransferase 1 restores mitochondrial oxidative phosphorylation in human trifunctional protein deficient fibroblasts

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ABSTRACT

Background: Mitochondrial Trifunctional Protein deficiency (TFPD) is a severe genetic disease characterized by altered energy metabolism and accumulation of long-chain (LC) acylcarnitines in blood and tissues. This accumulation could impair the mitochondrial oxidative phosphorylation (OxPhos), contributing to the non-optimal outcome despite conventional diet therapy with medium-chain triglycerides (MCT).

Method: Acylcarnitine and OxPhos parameters were measured in TFPD-fibroblasts obtained from 8 children and cultured in medium mimicking fasting (LCFA) or conventional treatment (MCT), with or without Etomoxir (ETX) an inhibitor of carnitine palmitoyltransferase 1 (CPT1) activity, and were compared to results obtained with fibroblasts from 5 healthy-control children. The effects of various acylcarnitines were also tested on control fibroblasts.

Results: In the LCFA-condition, TFPD-fibroblasts demonstrated a large accumulation of LC-acylcarnitines associated with decreased O_2-consumption (63 ± 3% of control, $P < 0.001$) and ATP production (67 ± 5%, $P < 0.001$) without modification of coupling efficiency. A dose-dependent decrease in O_2-consumption was reproduced in control fibroblasts by addition of increasing dose of LC-acylcarnitines, while it was almost preserved with MC-acylcarnitines. The MCT-condition reduced LC-acylcarnitine accumulation and partially improved O_2-consumption (80 ± 3%, $P < 0.01$) in TFPD-fibroblasts. The addition of ETX in both LCFA- and MCT-conditions normalized acylcarnitine profiles and restored O_2-consumption and ATP production at the same levels than control.

Conclusion: Accumulation of LC-acylcarnitines plays a major role in the pathophysiology of TFPD, reducing OxPhos capacities. These deleterious effects could be partially prevented by MCT-therapy and totally corrected by ETX. Inhibition of CPT1 may be view as a new therapeutic target for patients with a severe form of TFPD.

1. Introduction

Mitochondrial trifunctional protein (TFP) catalyzes the three final steps of mitochondrial long-chain (LC) fatty acid β-oxidation (FAO). The enzyme is composed of four α-subunits encoded by the *HADHA* gene, that contain the 2,3-long-chain enoyl-CoA hydratase and the long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) domains, and four β-subunits encoded by the *HADHB* gene, that contain the long-chain 3-ketoacyl-CoA thiolase (LKAT) domain [1]. The most common mutation c. 1528G > C of the α-subunit of the enzyme results in an isolated LCHAD deficiency (MIM#609016), without reduction in hydratase and thiolase activities, while other mutations in the *HADHA*

Abbreviations: BSA, bovine serum albumin; CPT1, carnitine palmitoyltransferase 1; DMEM, Dulbecco's modified eagle medium; ETX, etomoxir; FAO, fatty acid β-oxidation; LC, long-chain; LCFA, long-chain fatty acid; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; LKAT, long-chain 3-ketoacyl-CoA thiolase; MC, medium-chain; MCFA, medium-chain fatty acid; MCT, medium-chain triglycerides; OxPhos, oxidative phosphorylation; SRB, sulfurhodamine B; TFP, trifunctional protein; TFPD, trifunctional protein deficiency

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or *HADHB* genes are often responsible of a combined deficit of the 3 enzymes (MIM#609015). Only few cases of isolated LKAT deficiency have been reported [2,3].

Symptoms and prognosis of patients with TFP deficiency (TFPD) are heterogeneous, without a clear genotype-phenotype correlation [4–6]. Three clinical presentations have been described: a severe neonatal form with high rate mortality due to cardiomyopathy and Reye's syndrome, a moderate infantile hepatic presentation with episodes of hypoketotic hypoglycaemia, and a milder adolescent phenotype characterized by myopathy and episodes of rhabdomyolysis, especially in situation of intense lipid catabolism, such as prolonged fasting or infections [7–9]. Patients with isolated LCHAD deficiency could also develop peripheral neuropathy or retinopathy [10]. Biochemical disorders of TFPD are characterized by metabolic acidosis, hyperlactacidemia and accumulation of LC-acylcarnitines in tissues and blood [11–13]. The only treatment currently available is a diet limited in LC-fatty acids (LCFA) to prevent the accumulation of LCFA derivatives upstream the enzyme deficiency, and enriched in carbohydrates and medium-chain triglycerides (MCT) to restore energy production [14]. Unfortunately, this diet does not completely stop the disease progression with a high rate of mortality and morbidity related to the persistence of cardiac (cardiomyopathy, arrhythmia) or muscular (myopathy, rhabdomyolysis) involvement [15–17]. Some improvement have been reported with the use of heptanoate, an anaplerotic substrate for Krebs cycle, but these beneficial effects need to be further confirmed [18,19]. The physiopathology of TFPD remains poorly understood. However, secondary respiratory chain deficiencies have been recurrently reported in children with TFPD [7,20], suggesting that besides the reduced energy supply from LCFA, oxidative phosphorylation capacities may be impaired, possibly due to a toxic effect of LCFA derivatives accumulated upstream the enzyme deficiency [21–24]. To test this hypothesis, we have investigated the OxPhos capacities of TFPD and control fibroblasts cultured in various conditions and treated with a pharmacological inhibitor of carnitine palmitoyltransferase 1 (CPT1).

2. Material and methods

2.1. Reagents

Dulbecco's Modified Eagle's Medium (DMEM) 4.5 g/L glucose and 1 g/L glucose, fetal calf serum (FCS), Dulbecco's Phosphate Buffered Saline (DPBS) without calcium and magnesium, streptomycin/penicillin, and trypsin-EDTA were obtained from BioWhittaker® (Lonza Verviers, Belgium). Glucose free DMEM was purchased from Life Technologies (Invitrogen, Carlsbad, CA, USA). Medium-chain triglycerides (MCT) oil (mixture of MCT composed of hexanoic acid (1.7%), octanoic acid (58%), decanoic acid (38%), dodecanoic acid (1.7%) and tetradecanoic acid (0.6%)) was purchased from Nutricia® (Saint-Ouen, France). All other reagents were obtained from Sigma Chemicals (Sigma Aldrich, Lyon, France). LC and MC acylcarnitines were purchased from Dr. Herman J. Ten Brink, VU Medical Center (VU Medical Center, Amsterdam, Netherland), 3-hydroxylpalmitoylcarnitine was obtained from Pr Ernesto Brunet (Universidad Autonóma de Madrid, Madrid, Spain).

2.2. Patients

TFPD skin-fibroblast lines were obtained from 8 children with TFPD proved by enzyme activity measurement and genotype. The characteristics of these patients are summarized in Table 1. Three of them had a combined defect of the 3 enzymes, whereas the other had an isolated LCHAD deficiency due to the c.1528G > C mutation. Seven patients were homozygotes and one was compound heterozygous (patient 1). All of them presented severe clinical symptoms and were diagnosed before the age of 1 year. Half of the patients deceased in the months following diagnosis, while the others developed chronic symptoms (patients 3 and 8) or are still young (patients 5 and 6). Control fibroblasts were obtained from 5 children aged < 2 years and for whom FAO and oxidative phosphorylation were found to be normal. The study protocol was approved by the Ethics Committee in Human Research of Tours Hospital. All subjects and/or their parents had given their written informed consent.

2.3. Cell culture

Fibroblasts were cultured between passages 2 to 18 in DMEM supplemented with 10% FCS, 100 U/mL penicillin and 0.1 mg/mL streptomycin (cell growth medium), under standard conditions (5% CO_2, 37 °C). At 90% of cell confluence, medium was changed for the LCFA-medium (200 μM sodium palmitate bounded to bovine serum albumin (BSA) (molar ratio 6/1), DMEM 0.5 g/L glucose and 100 μM L-Carnitine), or MCT-medium (200 μM MCT oil, DMEM 1 g/L glucose and 100 μM L-carnitine), or LCFA + ETX-medium or MCT + ETX-medium obtained by addition of 100 μM Etomoxir in the previous media. After 48 h, the cells were washed twice with DPBS and harvested by trypsinization.

2.4. Determination of mitochondrial oxygen consumption and ATP synthesis

Mitochondrial oxygen consumption and ATP synthesis were measured according to the method of Peyta et al. [25], with slight modifications. Briefly, after trypsinization, cells were numbered with hemocytometer (Malassez, Marienfiled, Germany), centrifuged 3 min at 700*g*, and then suspended in a respiratory buffer without BSA (10 mM KH_2PO_4, 300 mM mannitol, 10 mM KCl, and 5 mM $MgCl_2$, pH 7.4). Cytoplasmic membrane permeabilisation was done by additionof 10 μg digitonin per million of cells during 2.5 min. The action of digitonin was stopped by additionof respiratory buffer with BSA. After a second centrifugation 2 min at 800*g*, cells were resuspended in 2 ml respiratory buffer with BSA, supplemented with 2 mM iodoacetate and 2 mM EDTA to prevent glycolytic ATP synthesis and ATP hydrolysis by cellular ATPases. Quality of permeabilisation was tested by addition of exogenous cytochrome C. ATP synthesis was started by the addition of 5 mM malate and 5 mM pyruvate (substrate for respiratory chain complex 1), and 10 mM succinate (substrate for respiratory chain complex II), and the subsequent addition of 1.5 mM adenosine diphosphate. The respiratory rates of 3.10^6 cells were recorded at 37 °C in 2 mL glass chamber using an oxygraph respirometer (Oxygraph +, Hansatech Instruments, Eurosep Instruments, Cergy, France). Aliquots were sampled every 40 s after quenching with an equal volume of 10% perchloric acid solution and neutralized by adding 25 mM 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid (HEPES), 2 mM EDTA, pH 7.75 buffer. The ATP synthesized in situ was measured using the Enliten ATP assay (Promega, Charbonnières-les-Bains, France). Luminescence was measured on a Glomax 20/20 luminometer (Promega, Charbonnières-les-Bains, France).

2.5. Acylcarnitine profile

One million-cell pellet was harvested after 48 h incubation in the different medium described above. The cell homogenate was extracted and analyzed for acylcarnitine content by electrospray MS-MS using an Api4500 tandem mass spectrometer (Sciex, Villebon sur Yvette, France). The values were expressed relative to protein content determined using the Pierce BCA protein assay kit (Thermo Scientific, Rockford, USA).