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Carnitine palmitoyltransferase 2: New insights on the substrate specificity and implications for acylcarnitine profiling

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ABSTRACT

Over the last years acylcarnitines have emerged as important biomarkers for the diagnosis of mitochondrial fatty acid β-oxidation (mFAO) and branched-chain amino acid oxidation disorders assuming they reflect the potentially toxic acyl-CoA species, accumulating intramitochondrially upstream of the enzyme block. However, the origin of these intermediates still remains poorly understood. A possibility exists that carnitine palmitoyltransferase 2 (CPT2), member of the carnitine shuttle, is involved in the intramitochondrial synthesis of acylcarnitines from accumulated acyl-CoA metabolites. To address this issue, the substrate specificity profile of CPT2 was herein investigated. *Saccharomyces cerevisiae* homogenates expressing human CPT2 were incubated with saturated and unsaturated C2–C26 acyl-CoAs and branched-chain amino acid oxidation intermediates. The produced acylcarnitines were quantified by ESI-MS/MS. We show that CPT2 is active with medium (C8–C12) and long-chain (C14–C18) acyl-CoA esters, whereas virtually no activity was found with short- and very long-chain acyl-CoAs or with branched-chain amino acid oxidation intermediates. *Trans*-2-enoyl-CoA intermediates were also found to be poor substrates for CPT2. Inhibition studies performed revealed that *trans*-2-C16:1-CoA may act as a competitive inhibitor of CPT2 (K_i of 18.8 μM). The results obtained clearly demonstrate that CPT2 is able to reverse its physiological mechanism for medium and long-chain acyl-CoAs contributing to the abnormal acylcarnitines profiles characteristic of most mFAO disorders. The finding that *trans*-2-enoyl-CoAs are poorly handled by CPT2 may explain the absence of *trans*-2-enoyl-carnitines in the profiles of mitochondrial trifunctional protein deficient patients, the only defect where they accumulate, and the discrepancy between the clinical features of this and other long-chain mFAO disorders such as very long-chain acyl-CoA dehydrogenase deficiency.

1. Introduction

Mitochondrial fatty acid β-oxidation (mFAO) is the most important source of energy, especially for high-energy demanding tissues such as the heart and skeletal muscle [1]. In order to be metabolized in the mitochondria, long-chain fatty acids must first undergo activation, prior to its transport into the mitochondrial matrix. The import of activated long-chain fatty acids (long-chain acyl-CoAs) into the mitochondrial matrix is handled by the carnitine shuttle. This system operates by the combined action of carnitine palmitoyltransferase 1 (CPT1, EC 2.3.1.21), carnitine/acylcarnitine translocase (CACT, SLC25A20) and carnitine palmitoyltransferase 2 (CPT2, EC 2.3.1.21) and requires the presence of L-carnitine. Long-chain acyl-CoA esters are first converted into the corresponding carnitine esters by CPT1, followed by transport of the resulting acylcarnitines across the mitochondrial membrane by CACT, located in the inner mitochondrial membrane, in exchange with free carnitine. The final step of this cycle, catalyzed by CPT2, reconverts the acylcarnitines back into the respective acyl-CoA esters that can then undergo β-oxidation [2–4].

During fasting or when the energy demand is increased, fatty acid oxidation is crucial for cellular energy homeostasis. Pathologies involving one or several defects of the mitochondrial fatty acid β-oxidation system, especially those concerning long-chain fatty acids, are complex and clinically heterogeneous. Affected patients usually present hypoketotic hypoglycemia with hepatic, cardiac and muscular symptoms [5,6]. In

Abbreviations: mFAO, mitochondrial fatty acid β-oxidation; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; CACT, carnitine/acylcarnitine translocase; MTP, mitochondrial trifunctional protein; LCHAD, long-chain 3-hydroxyacyl-CoA dehydrogenase; VLCAD, very long-chain acyl-CoA dehydrogenase; DMN, dimethylnonanoate; DMH, dimethylheptanoate; Prist-CoA, Pristanoyl-CoA

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addition to the symptoms usually observed in mFAO disorders, those affecting long-chain mFAO such as deficiencies at the level of very long-chain acyl-CoA dehydrogenase (VLCAD), long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) tend to be more severe and present unusual features. Such complications may be caused by the accumulation of toxic intermediates as a consequence of the impaired β-oxidation of long-chain fatty acids [6,7]. An impairment in mFAO, as in genetic FAO deficiencies, gives rise to the intracellular accumulation of acyl-CoAs and its β-oxidation intermediates, which may be further metabolized by alternative oxidative pathways, such as ω and ω − 1 oxidation, leading to the production of dicarboxylic acids and/or elimination as acylglycines and/or acylcarnitines with secondary L-carnitine depletion. Acylcarnitines are currently used in neonatal screening programs as biomarkers for the diagnosis of mFAO disorders. Nevertheless, the etiology of these acylcarnitines is still not completely elucidated. It is usually considered that the observed acylcarnitine profiles reflect the intramitochondrially accumulating acyl-CoAs which are exported out of the mitochondria as their correspondent carnitine esters [8,9].

It is hypothesized that the acylcarnitines are formed by carnitine palmitoyltransferase 2, although formal proof is lacking for most of the acyl-CoAs. This would be followed by the export of these acylcarnitines from the mitochondria and the cell, processes not yet definitively clarified. Some work has been done pointing towards reversibility of the physiologic mechanism of the carnitine shuttle [10,11]. However the lack of a comprehensive study on this subject and specifically on human CPT2 prompted us to investigate the complete substrate specificity of this enzyme. The data described in this paper provides new insights into the specific role of CPT2 in the export of toxic acyl-CoAs from the mitochondria into the cytosol and subsequently into the extracellular space.

2. Materials and methods

2.1. Strains of Saccharomyces cerevisiae and growth media

The Δ*cat2* (*cat2::*KAN) and Δ*fox2* (*fox2::*KAN) deletion mutants of *S. cerevisiae* strain BY4742 (*Matα*; *his3Δ1*; *leu2Δ0*; *lys2Δ0*; *ura3Δ0*) were used. The strain was cultured in rich glucose medium, YPD (glucose 20 g/l, peptone 20 g/l and yeast extract 10 g/l) and minimal glucose medium, YNBD (glucose 3 g/l or 20 g/l and yeast nitrogen base without amino acids 6.7 g/l). For plates, agar 20 g/l was added. Galactose medium contained yeast extract 1 g/l, yeast nitrogen base without amino acids 6.7 g/l and galactose 200 g/l. Amino acids were added (2 mg/ml) as required. Yeast nitrogen base, yeast extract, peptone and agar were obtained from Difco Laboratories Inc. (Detroit, MI). Glucose and galactose were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.2. Plasmids and cell culture conditions

The plasmid used (pYES2-CPT2) was a generous gift from Dr. F. Taroni, Milano, Italy [12]. Confirmation of the sequence was achieved by direct sequencing which showed a correct insertion into the pYES2 vector of the complete open reading frame of the human CPT2, including the region coding for the corresponding mitochondrial targeting signal. Transformation of the Δ*cat2* and Δ*fox2* mutants with the pYES2-CPT2 plasmid was performed using the lithium acetate method, as described elsewhere [13]. Transformed cells were harvested by centrifugation, spread on 3 g/l YNBD plates containing amino acids as required and cultured for 2 days at 28 °C.

2.3. Growth conditions and preparation of yeast homogenates

Cells were grown on minimal 20 g/l glucose medium for at least 24 h at 225 rpm and 28 °C in a gyro shaker and then shifted by centrifugation to galactose medium. Cells from overnight cultures grown on galactose medium were harvested and treated with zymolyase as described elsewhere [14]. The resulting protoplasts were homogenized by sonication (three times, 10 s at 8 W) on ice and suspended in PBS with Complete^{mini} tablets containing a cocktail of protease inhibitors (Roche; Basel, Switzerland). Protein concentration of the yeast homogenates was determined using the bicinchoninic acid assay (BCA, Sigma-Aldrich) [15] and human serum albumin as a reference substance.

2.4. Acyl-CoA esters preparation

Trans-2-dodecenoyl-CoA (C12:1-CoA) and *trans*-2-hexadecenoyl-CoA (C16:1-CoA), were enzymatically synthesized from the corresponding saturated CoA esters using acyl-CoA oxidase. *Cis*-5-tetradecenoyl-CoA (C14:1-CoA) was synthesized as described by Rasmussen et al. [16]. Pristanoyl-CoA, 4,8-dimethylnonanoyl-CoA and 2,6-dimethylheptanoyl-CoA were synthesized by Prof. Dr. G. Dacremont, Belgium. All other CoA esters were obtained from Sigma-Aldrich.

2.5. Determination of carnitine palmitoyltransferase 2 activity using different acyl-CoA esters

Carnitine palmitoyltransferase 2 activity was determined using the method described by van Vlies et al. [17]. The standard mixture contained 150 mM potassium chloride, 25 mM Tris–HCl pH 7.4, 2 mM EDTA, 10 mM potassium phosphate buffer pH 7.4, 1 mg/ml bovine serum albumin (BSA) essentially fatty acid free, 500 μM L-carnitine and 25 μM of each acyl-CoA ester to a final volume of 150 μl. The reaction was initiated by the addition of 20 μl of sample (*S. cerevisiae* homogenate) and was allowed to proceed at 37 °C. After 10 min incubation, the reaction was terminated by adding 750 μl acetonitrile containing 50 pmol d3C3-, 50 pmol d3C8- and 25 pmol d3C16-carnitine internal standards. After derivatization of the produced acylcarnitines with 1-propanol/acetylchloride 4/1 (v/v), these intermediates were quantified by Electro Spray Ionization Tandem Mass Spectrometry (ESI-MS/MS). Negative controls were performed as described above, using yeast homogenates transformed with the empty vector.

2.6. Inhibition studies upon carnitine palmitoyltransferase 2 activity

The effect of *trans*-2-C16:1-CoA on CPT2 activity was determined by measuring its activity in the presence of different concentrations of this compound (0–20 μM) and using C16-CoA as substrate (0–40 μM). Activity was measured as described above with some modifications. After 5 min incubation at 37 °C the reaction was terminated by adding 750 μl acetonitrile containing 100 pmol d3C3-, 100 pmol d3C8- and 50 pmol d3C16-carnitine internal standards. In order to gain 10 times more sensitivity, the samples were analyzed on UPLC-MS/MS without derivatization.

3. Results

3.1. Determination of carnitine palmitoyltransferase 2 activity using different acyl-CoA esters

In order to determine the substrate specificity of human CPT2, we transformed the Δ*cat2 S. cerevisiae* mutant (BY4742 *cat2::*KAN) with a plasmid expressing human CPT2 (see Section2.2). This mutant has no carnitine acetyltransferase activity (cat2, converting acetyl-CoA into acetylcarnitine). Immunoblot analysis after subcellular fractionation on Nycodenz gradient showed that the protein is localized in the mitochondria, although not fully processed to maturity (results not shown). More important, the heterologously expressed human CPT2 is in its active form and thus used for subsequent kinetic measurements. The results depicted in Fig. 1A show that CPT2 is well expressed and is active towards medium (C8–C12) and long-chain (C14–C18) acyl-CoA esters. Virtually no activity was found with short-