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Carnitine Palmitoyltransferase 2: New Insights on the Substrate Specificity and Implications for Acylcarnitine Profiling

Sara Violante^{a,b}, Lodewijk IJlst^b, Henk van Lenthe^b, Isabel Tavares de Almeida^a, Ronald J. Wanders^{b*} and Fátima V. Ventura^{a*#}

^a *Metabolism and Genetics Group, Research Institute for Medicines and Pharmaceutical Sciences, iMed.UL, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal*

^b *Laboratory Genetic Metabolic Diseases, Department Clinical Chemistry and Pediatrics, University of Amsterdam, Academic Medical Center, Meibergdreef 9, 1105 AZ Amsterdam, The Netherlands*

* These authors should be considered as equal last authors.

Corresponding author: Metabolism and Genetics Group, Research Institute for Medicines and Pharmaceutical Sciences, iMed.UL, Faculdade de Farmácia, Universidade de Lisboa, Av. Prof. Gama Pinto, 1649-003 Lisboa, Portugal. Tel: +351 21 7946400; Fax: +351 21 7946491; *E-mail*: fatima.ventura@ff.ul.pt

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

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 aminoacid 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 aminoacid 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 μ M). 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.

Keywords: Acylcarnitines; Carnitine palmitoyltransferase 2 (CPT2); Long-chain acyl-CoA metabolites; Fatty acid oxidation (FAO); Mitochondrial Trifunctional Protein (MTP)

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 hypoglycaemia with hepatic, cardiac and muscular symptoms [5, 6]. In 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 aetiology 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 $\Delta cat2$ (*cat2::KAN*) and $\Delta fox2$ (*fox2::KAN*) deletion mutants of *S. cerevisiae* strain BY4742 (*Mata*; *his3 Δ 1*; *leu2 Δ 0*; *lys2 Δ 0*; *ura3 Δ 0*) 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 aminoacids 6.7 g/L). For plates, agar 20 g/L was added. Galactose medium contained yeast extract 1 g/L, yeast nitrogen base without aminoacids 6.7 g/L and galactose 200 g/L. Aminoacids 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 $\Delta cat2$ and $\Delta fox2$ 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 aminoacids as required and cultured for two 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 (3 times, 10 s at 8W) 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, St. Louis, MO, USA) [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 and co-workers [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 (St. Louis, MO, USA).

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 and co-workers [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 μ M L-carnitine and 25 μ M of each acyl-CoA ester to a final volume of 150 μ L. The reaction was initiated by the addition of 20 μ L of sample (*S. cerevisiae* homogenate) and was allowed to proceed at 37°C. After 10 min incubation, the reaction was terminated by adding 750 μ L 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 μ M) and using C16-CoA as substrate (0 – 40 μ M). 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 analysed 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 $\Delta cat2$ *S. cerevisiae* mutant (BY4742 *cat2::KAN*) with a plasmid expressing human CPT2 (see section 2.2). 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- and very long-chain acyl-CoA esters (Fig. 1A). Furthermore, CPT2 showed activity towards the *cis*-5 and *cis*-9 unsaturated acyl-CoAs tested (Fig. 1B) and the mitochondrial β -oxidation intermediates 3-OH and 3-keto-palmitoyl-CoA (Fig. 1C). Surprisingly, different *trans*-2-enoyl-CoA, which are intermediates of mFAO, including *trans*-2-C16:1-CoA and *trans*-2-C12:1-CoA, were found to be poor substrates for CPT2. We observed that *trans*-2-C16:1 and *trans*-2-C12:1-CoA intermediates show 1.5% and 1% activity respectively when compared with the straight-chain acyl-CoAs intermediates of the same chain-length (C16-CoA and C12-CoA). In the same manner, only a small percentage of activity (approximately 1.5%) was found when comparing with other enoyl substrates (*cis*-5 and *cis*-9-C14:1-CoA) (Fig. 1B).

Breakdown of different aminoacids including the branched-chain amino acids also involves CoA esters. When tested, none of these acyl-CoAs were found to be a substrate for CPT2 (see Table 1). Intermediates from peroxisomal fatty acid β -oxidation, namely 4,8-dimethylnonanoyl-CoA (DMN-CoA), 2,6-dimethylheptanoyl-CoA (DMH-CoA) and pristanoyl-CoA, were also investigated as potential substrates for CPT2. Interestingly, CPT2 was found to be active with DMN-CoA which is in line with data in literature [18], whereas virtually no activity was found with the substrates pristanoyl-CoA or DMH-CoA (Fig. 1D).

3.2. Determination of the enzymatic kinetic parameters of CPT2

In order to resolve whether *trans*-2-enoyl-CoAs may act as inhibitors of CPT2 activity, we determined the kinetic parameters of CPT2 (K_m and V_{max}) using C16-CoA and *trans*-2-C16:1-CoA as substrates. Employing the Lineweaver-Burk linearization of the Michaelis-Menten equation, we found that although *trans*-2-C16:1-CoA and C16-CoA have approximately the same affinity for CPT2 ($K_{m(C16-CoA)} = 7.1 \mu\text{M}$ and $K_{m(trans-2-C16:1-CoA)} = 8.1 \mu\text{M}$), the catalytic efficiency (here expressed as the ratio V_{max}/K_m) for C16-CoA is approximately 20-fold higher than for *trans*-2-C16:1-CoA (Table 2). These results indicate that *trans*-2-enoyl-CoAs might act as inhibitors of CPT2.

3.3. Inhibition studies with *trans*-2-C16:1-CoA

To verify if *trans*-2-C16:1-CoA may act as an inhibitor of the conversion of acyl-CoA esters into carnitine derivatives catalysed by CPT2, inhibition studies were performed in the $\Delta fox2$ *S. cerevisiae* mutant (BY4742 *fox2::KAN*) transformed with the plasmid described in section 2.2 from Materials and Methods. This mutant lacks 2-enoyl-CoA hydratase activity (*fox2*, converting *trans*-2-enoyl-CoA esters into L-3-hydroxyacyl-CoA esters). The results shown for each concentration of inhibitor reveal a small variation (6%) concerning the V_{max} while an increase of approximately 30% in the K_m is observed. This suggests that *trans*-2-C16:1-CoA acts as a competitive inhibitor of CPT2 with a K_i of 18.8 μM (Fig. 2 and Table 3).

4. Discussion

The work described in this paper clearly shows that CPT2, when operating in the reverse direction (acyl-CoA + carnitine \rightarrow acylcarnitine + CoA), accepts, at least in vitro, a range of different medium and long-chain straight-chain acyl-CoAs (Fig. 1A) and converts them into the respective acylcarnitines. These results are in agreement with the data reported by Schaefer *et al.* [11] in permeabilized human fibroblasts. In this model of study the substrate specificity of a malonyl-CoA insensitive acyltransferase, identified as CPT2, differs to some extent from the results obtained by us using recombinant human CPT2. According to these authors, CPT2 shows higher activity with substrates ranging from C8 to C16-CoA with C12-CoA as the preferred substrate followed by C8-CoA, which was also recognized as the preferred substrate for the recombinant rat liver CPT2 studied by Johnson *et al.* [19]. Our results, however, show that CPT2 has specificity towards longer acyl-CoA esters ranging from C8 to C20-CoA with C10 to C14-CoA as the preferred substrates (Fig. 1A), which compares well previous data on CPT2 in bovine liver mitochondria [20], chick embryo liver [21] and beef heart [22].

Concerning the possible conversion of mFAO intermediates into their corresponding acylcarnitine esters by CPT2 we observed that this enzyme accepts different mFAO intermediates as substrates, including 3-OH and 3-keto acyl-CoA esters, though less efficiently (Fig. 1C). Nevertheless, *trans*-2-enoyl-intermediates were found to be poor substrates for the enzyme which suggests that CPT2 may not be as active towards *trans*-2-enoyl-CoAs as shown for the other mFAO intermediates or other unsaturated acyl-CoA esters (Fig. 1B). This data is consistent with our previous work using purified human CPT2 protein [10]. The *trans* double bond in the *trans*-2-enoyl-CoA esters may cause a spatial constraint in the binding of these intermediates to the active site of the enzyme causing the reaction to occur slower than with other substrates. These findings may well explain the fact that *trans*-2-enoyl-carnitine intermediates have never been reported neither in plasma nor in fibroblasts acylcarnitine profiles from patients with mFAO disorders [8]. The catalytic efficiency of CPT2 with the substrates *trans*-2-enoyl-CoAs is much lower than when handling the substrate palmitoyl-CoA (Table 2) and therefore the reaction is expected to proceed very slowly. Accordingly, enoyl intermediates were found

to act as competitive inhibitors of CPT2 activity (Table 3; Fig. 3). This may lead to the accumulation of such intermediates within the mitochondrial matrix in specific mFAO deficiencies, which would block further clearance of other toxic acyl-CoA species accumulating in these disorders. *Trans*-2-enoyl-CoAs are known toxic intermediates [23-25] which are expected to accumulate intramitochondrially in mitochondrial trifunctional protein (MTP) and long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) deficiencies. In addition to the symptoms usually observed in all mFAO disorders, MTP and LCHAD deficient patients also present severe characteristic symptoms including neurological and ophthalmological manifestations (peripheral neuropathy and retinopathy) and serious maternal complications during pregnancy [25-28]. It is unlikely that such severe abnormalities result from the simple accumulation of long-chain intermediates as they do not emerge in patients suffering from other long-chain mFAO disorders such as a deficiency in very long-chain acyl-CoA dehydrogenase (VLCAD) [29]. Such clinical facts suggest different pathological mechanisms underlying these disorders. This may in part be explained by the potential inhibition of CPT2 by *trans*-2-enoyl-CoAs which only accumulate in MTP and LCHAD deficiencies.

CPT2 activity was also evaluated with some peroxisomal FAO substrates and intermediates. Only 4,8-dimethylnonanoyl-CoA (DMN-CoA) shows reactivity with the enzyme (Fig. 1D). This is in line with the pristanic acid degradation route, where the first steps are known to take place in the peroxisome [18]. Peroxisomal degradation of pristanic acid proceeds for three cycles of β -oxidation yielding the intermediate DMN-CoA, probably converted by carnitine octanoyltransferase into the respective carnitine ester [30] and further exported into the cytoplasm. Further oxidation requires the mitochondrial import of DMN-carnitine and its conversion back into DMN-CoA. One additional cycle of β -oxidation in mitochondria then leads to the formation of 2,6-dimethylheptanoyl-CoA (DMH-CoA) [31]. The metabolic fate of DMH-CoA is not known [31] but its destiny, according to our results, does not entail its conversion to the corresponding carnitine ester, at least as catalyzed by CPT2. The importance of these data for the *in vivo* situation remains to be resolved especially since neither DMN nor DMH carnitine derivatives have ever been reported among the acylcarnitine profiles of any

mFAO defect whose block should lead to the concomitant accumulation of mitochondrially metabolized peroxisomal FAO intermediates.

Also several acyl-CoA esters formed during branched-chain aminoacid oxidation were tested and were not found to be substrates for CPT2. The presence of the corresponding carnitine intermediates suggests that these may be formed by the action of other transferases, including carnitine acetyltransferase (CRAT). A more detailed study on the substrate specificity of CRAT is lacking in literature, thus requiring further investigation in future work.

5. Conclusions

In summary although acylcarnitines have been recognized over the last years as important biomarkers for the early detection of mitochondrial [32-37] and peroxisomal [18] fatty acid oxidation disorders, peroxisomal biogenesis defects [38] and branched-chain aminoacid oxidation disorders [32] and acylcarnitine profiling in blood allows a rapid and effective screening of the aforementioned inborn errors of metabolism, the studies presented here clearly demonstrate that the profile of acylcarnitines as observed in plasma may not reflect the profile of acyl-CoA species which accumulate within the mitochondrial matrix under certain conditions, at least not fully. If an intermediate containing a *trans*-2 double bond accumulates intramitochondrially, the respective carnitine esters might be missed in the diagnosis. Hence, the data gathered in the present paper support the rationale for the relevance of determining mitochondrial acyl-CoA esters profile [39]. Undoubtedly this approach may be crucial for the elucidation of the diagnosis and further treatment of mFAO disorders as well as for the understanding of the pathogenic mechanisms involved in these inborn errors of metabolism.

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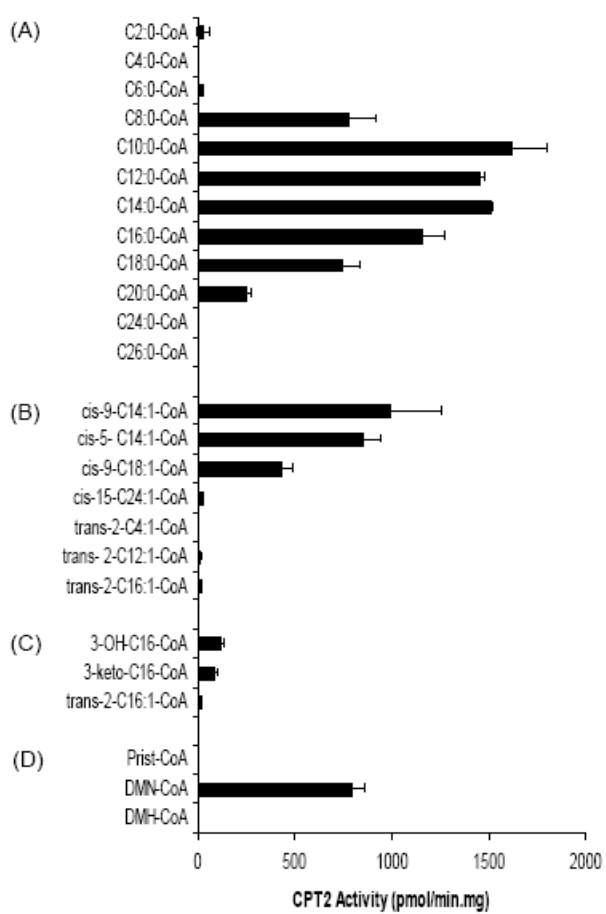
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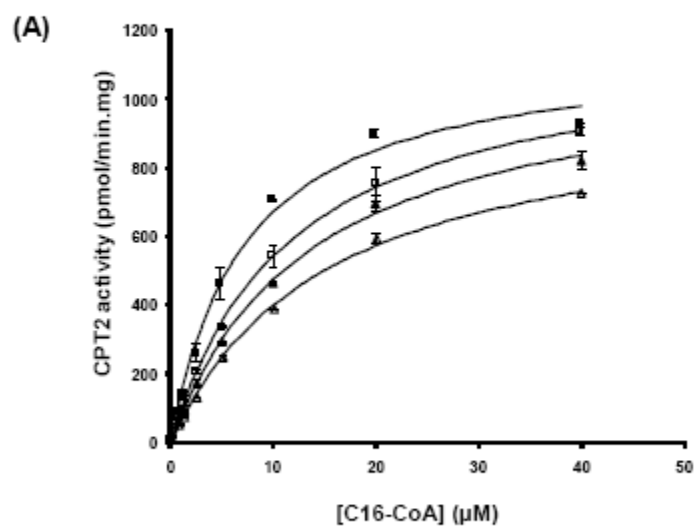
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Fig. 1



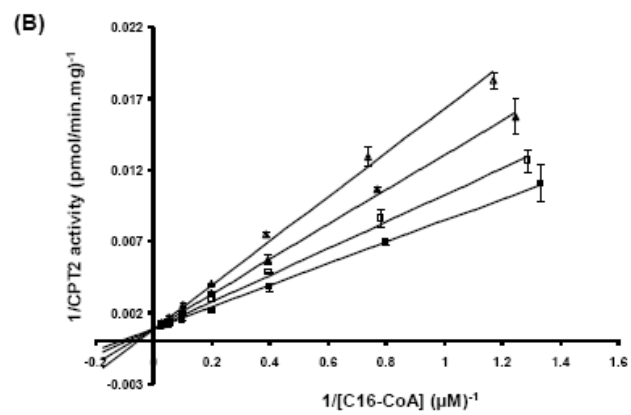
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Fig. 2A



ACCEPTED

Fig. 2B



ACCEPTED MANUSCRIPT

Table 1

Evaluation of CPT2 activity with acyl-CoAs formed upon branched-chain aminoacids oxidation. Activity is expressed as percentage of control (C16-CoA was used as a control substrate)

CoA-ester	CPT2 activity (% of control)
C16-CoA	100
isovaleryl-CoA	<0.1
isobutyryl-CoA	<0.1
acetoacetyl-CoA	<0.1
DL-3-hydroxybutyryl-CoA	<0.1
3-hydroxyisobutyryl-CoA	<0.1
2-methylacetoacetyl-CoA	<0.1
methylcrotonyl-CoA	<0.1
glutaryl-CoA	<0.1
3-hydroxy-3-methylglutaryl-CoA	<0.1
2-methylbutyryl-CoA	<0.1
2-methyl-3-hydroxybutyryl-CoA	<0.1

Table 2

Kinetic constants and catalytic efficiency (V_{\max}/K_m) of CPT2 with C16:0-CoA and *trans*-2-C16:1-CoA as substrates. The reactions were measured at 37°C for 5 min in a standard incubation medium described in Materials and Methods with different concentrations of the acyl-CoA esters. The produced acylcarnitines were quantified after derivatization with 1-propanol/acetylchloride by ESI-MS/MS. Values were calculated from Lineweaver-Burk plots (not shown).

	C16:0-CoA	<i>trans</i> -2-C16:1-CoA
$K_{m_{app}}$ (μ M)	7.1	8.1
$V_{max_{app}}$ (pmol/min.mg)	1156	77
V_{max}/K_m	163	9.5

Table 3

Kinetic parameters K_m and V_{max} of CPT2 for C16:0-CoA as substrate with different *trans*-2-C16:1-CoA concentrations. The K_i value for CPT2 with *trans*-2-C16:1-CoA was 18.8 μM . The reactions were measured at 37°C for 5 min in a standard incubation medium described in Materials and Methods with C16:0-CoA from 0 to 40 μM . The produced acylcarnitines were quantified by UPLC-MS. Values were calculated from non-linear regression plots using the Berkley Madonna™ software.

	<i>Trans</i> -2-C16:1-CoA			
	0 μM	5 μM	10 μM	20 μM
K_m^{app} (μM)	7.1	11.6	13.5	15.0
V_{max}^{app} (pmol/min.mg)	1156	1175	1120	1004