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NOVEL ASPECTS OF CARNITINE FUNCTION AND METABOLISM

Sara Liliana Nunes Violante

DOUTORAMENTO EM FARMÁCIA

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Novos Aspectos da Função e Metabolismo da Carnitina

Dissertação apresentada à Faculdade de Farmácia da Universidade de Lisboa para obtenção do grau de Doutor em Farmácia (especialidade Bioquímica)

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Para os meus Pais e Irmão

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Abbreviations

3MGH	3-Methylglutaconyl-CoA hydratase
3-OH-C16-	3-Hydroxypalmitoyl-
γ-BB	4-*N*-Trimethylaminobutyrate; γ-Butyrobetaine
γ-BBD	γ-Butyrobetaine dioxygenase
ABC	ATP-binding cassette
ABCD	ABC transporter
ACOX	Acyl-CoA oxidase
ACS	Acyl-CoA synthetase
ACSL	Long-chain acyl-CoA synthetase
ADP	Adenosine 5'-diphosphate
AFLP	Acute Fatty Liver of Pregnancy
ALDP	Adrenoleukodystrophy protein (or ABCD1)
ALDRP	ALD-related protein (or ABCD2)
ASD	Autism spectrum disorders
ASP	Acid soluble products
ATP	Adenosine 5'-triphosphate
BCAA	Branched-chain amino acid
BCAAO	Branched-chain amino acid oxidation
BSA	Bovine serum albumin
C10:0	Decanoic acid; capric acid
C12:0	Dodecanoic acid; lauric acid
C4:1-	*Trans*-2-butenoyl-; *trans*-2-ene-C_4-; crotonyl-
C5:1-	*Trans*-2-methyl-2-butenoyl; *trans*-2-ene-2-methyl-C_4-; tiglyl-
C12:1-	*Trans*-2-dodecenoyl-
C16:1-	*Trans*-2-hexadecenoyl-
CACT	Carnitine/acylcarnitine translocase
C*n*-carnitine	Acyl residue with *n* carbons esterified with carnitine
C*n*-CoA	Acyl residue with *n* carbons esterified with CoA
CoA	Coenzyme A
CPT1	Carnitine palmitoyltransferase 1
CPT2	Carnitine palmitoyltransferase 2
CrAT	Carnitine acetyltransferase
CrOT	Carnitine octanoyltransferase
CS	Citrate synthase
DBP	D-bifunctional protein
DCA	Dicarboxylic acid

DHCA	Dihydroxycholestanoic acid
DMH	2,6-Dimethylheptanoyl-
DMN	4,8-Dimethylnonanoyl-
DTNB	5,5'-Dithiobis-(2-nitrobenzoic acid)
EDTA	Ethylene diamine tetraacetic acid
ESI-MS/MS	Electrospray ionization tandem mass spectrometry
ETF	Electron-transfer flavoprotein
FABPpm	Fatty acid binding protein, isoform from plasma membrane
FAD	Flavin adenine dinucleotide (oxidized)
$FADH_2$	Flavin adenine dinucleotide (reduced)
FAO	Fatty acid oxidation
FAT/CD36	Fatty acid translocase
FATP	Fatty acid transport protein
FBS	Fetal bovine serum
GABA	4-Aminobutyric acid
HELLP	Hemolysis, Elevated Liver enzymes, Low Platelets
HIBADH	3-Hydroxyisobutyrate dehydrogenase
HIBCH	3-Hydroxyisobutyryl-CoA dehydrogenase
HMG	3-Hydroxy-3-methylglutaryl-
HPLC	High-performance liquid chromatography
HTML	3-Hydroxy-6-*N*-trimethyllysine
HTMLA	HTML aldolase
HSA	Human serum albumin
IBD	Isobutyryl-CoA dehydrogenase
IMM	Inner mitochondrial membrane
IMS	Intermembrane space
IVD	Isovaleryl-CoA dehydrogenase
*K*i	Inhibition constant
*K*m	Michaelis-Menten constant
L-AC	L-Aminocarnitine
LBP	L-Bifunctional protein
LCAD	Long-chain acyl-CoA dehydrogenase
LCEH	Long-chain enoyl-CoA hydratase
LCFA	Long-chain fatty acid
LCHAD	Long-chain 3-hydroxyacyl-CoA dehydrogenase
LKAT	Long-chain 3-ketoacyl-CoA thiolase
MADD	Multiple acyl-CoA dehydrogenation defect
MCAD	Medium-chain acyl-CoA dehydrogenase

MCFA	Medium-chain fatty acid
MCC	3-Methylcronotyl-CoA carboxylase
MCT	Medium-chain triglycerides
MES	2-[*N*-morpholino]ethanesulfonic acid
mFAO	Mitochondrial fatty acid β-oxidation
mFAOD	Mitochondrial fatty acid β-oxidation disorders
MHBD	2-Methyl-3-hydroxybutyryl-CoA dehydrogenase
MKAT	Medium-chain 3-ketoacyl-CoA thiolase
MOPS	3-[*N*-morpholino]propanesulfonic acid
MS/MS	Tandem mass spectrometry
MTP	Mitochondrial trifunctional protein
NAD^+	Nicotinamide adenine dinucleotide (oxidized)
NADH	Nicotinamide adenine dinucleotide (reduced)
NDA	Non-dysmorphic autism
OCTN2	Organic cation/carnitine Na^+-dependent transporter
OMM	Outer mitochondrial membrane
PBS	Phosphate buffer saline
PDHc	Pyruvate dehydrogenase complex
PDK	Pyruvate dehydrogenase kinase
PEX	Peroxisomal biogenesis factor
PMP70	70-kDa Peroxisomal membrane protein (or ABCD3)
PMP70R	PMP70-related protein
POCA	2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate
PPARα	Peroxisome proliferator activated receptor alpha
PPARδ	Peroxisome proliferator activated receptor delta
Prist-	Pristanoyl-
SBCAD	Short branched-chain acyl-CoA dehydrogenase
SCAD	Short-chain acyl-CoA dehydrogenase
SCHAD	Short-chain 3-hydroxyacyl-CoA dehydrogenase
SEM	Sucrose/EDTA/MOPS
shRNA	short hairpin RNA
SKAT	Short-chain 3-ketoacyl-CoA thiolase
SPC	Sterol carrier protein
SPCx	SPC-2/3-ketoacyl-CoA thiolase
TH	Thioesterase
THCA	Trihydroxycholestanoic acid
TMABA	4-Trimethylaminobutyraldeyde
TMABA-DH	TMABA dehydrogenase

TML	6-*N*-Trimethyllysine
TMLD	Trimethyllysine dioxygenase
TMLHE	Trimethyllysine hydroxylase, epsilon
UPLC-MS/MS	Ultra-performance liquid chromatography tandem mass spectrometry
VDAC	Voltage dependent anion channel
VLCAD	Very long-chain acyl-CoA dehydrogenase
*V*max	Maximum velocity

Summary

Normal functioning of fatty acid oxidation and energy metabolism depends on carnitine. Although this compound has been extensively studied over the years, some aspects of carnitine metabolism and function remain unclear. This thesis aimed to: 1) elucidate the reverse action of the carnitine shuttle in the mitochondrial detoxification of intermediates accumulating in mitochondrial fatty acid β-oxidation (mFAO) and branched-chain amino acid (BCAA) oxidation disorders; 2) shed light on how acylcarnitine intermediates cross the outer mitochondrial membrane; 3) clarify the contribution of peroxisomes to the oxidation of medium-chain fatty acids when the carnitine shuttle is impaired, and 4) give a new insight on the importance of carnitine and the carnitine biosynthesis pathway in autism spectrum disorders.

Deficiencies in the mFAO or BCAA oxidation pathways generally lead to the intramitochondrial accumulation of acyl-coenzyme A (CoA) esters. These acyl-CoAs are potentially toxic to the mitochondria and to the cell and are, therefore, readily conjugated with other molecules and converted into compounds that can then undergo mitochondrial and cellular export. The production of acylcarnitines is one of the mechanisms used to detoxify the mitochondria and the cell from the accumulating acyl-CoAs. Using *in vitro* techniques we have thoroughly studied the substrate specificity of two acyltransferases generally assumed to be responsible for the mitochondrial synthesis of acylcarnitines. We have shown that carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltransferase are responsible for the production of most of the acylcarnitines commonly found in mFAO disorders or deficiencies affecting the BCAA oxidation. Furthermore, we observed that *trans*-2-enoyl-CoAs are poor substrates for both acyltransferases and even act as CPT2 inhibitors, possibly by interfering with the catalytic mechanism of the enzyme. This may explain not only the absence of some of the respective *trans*-2-enoyl-carnitine intermediates from plasma acylcarnitine profiles of patients affected with a mFAO or BCAA oxidation disorder, but also the severity associated with such pathologies, as *trans*-2-enoyl-CoAs may additionally interfere with the metabolism of other compounds.

It was also important to clarify the mechanism by which acylcarnitnes are exported to the cytosol and out of the cell. Using cell lines with deficiencies in specific proteins associated with the carnitine transport and in the presence and absence of a deficiency in the medium-chain acyl-CoA dehydrogenase enzyme, induced by gene silencing, we have confirmed the *in vitro* results obtained for recombinant CPT2 and showed that, in intact cells, CPT2 is responsible for the production of medium-chain acylcarnitines. Moreover, we also observed that carnitine/acylcarnitine translocase (CACT) is the protein responsible for the mitochondrial export of medium-chain acylcarnitines (through the inner mitochondrial membrane) and that the cellular export of these intermediates does not seem to rely on the plasma membrane carnitine transporter, OCTN2. In the same context, the mechanism by which acylcarnitines cross the outer mitochondrial

membrane (OMM) is still far from being elucidated. It has been suggested that carnitine palmitoyltransferase 1 (CPT1) forms oligomeric complexes in the OMM. This would result in the formation of a hexameric structure that could function as a pore, facilitating the transport of acylcarnitines across the OMM. We have demonstrated that CPT1 does not seem to be crucial in the passage of acylcarnitines through the OMM for further oxidation within the mitochondria, or at least this is not the exclusive route for the access of acylcarnitines to the intermembrane space.

It is generally assumed that medium-chain fatty acids (MCFAs) are oxidized in mitochondria independently from carnitine. However, the true contribution of the carnitine shuttle to the oxidation of MCFAs has remained elusive. We show that lauric acid, a MCFA, also depends on the carnitine shuttle to be fully oxidized in the mitochondria. Furthermore, we observed that when the carnitine shuttle is impaired, this MCFA is able to undergo one or two cycles of peroxisomal β-oxidation. This shows that peroxisomes may have a more prominent role than earlier assumed in the oxidation of straight-chain fatty acids which are not typically metabolized in this cellular compartment, potentially acting as a compensatory mechanism when mFAO is compromised.

Finally, we show that carnitine and its biosynthetic pathway may have an important role in neurologic development. In patients with non-dysmorphic autism, we found a common deletion in exon 2 of the *TMLHE* gene, encoding the first enzyme of the carnitine biosynthesis pathway, trimethyllysine dioxygenase. This leads to the absence of protein and accumulation of trimethyllysine in the plasma and urine of patients. We have therefore characterized the first inborn error of carnitine biosynthesis and found that this defect is associated with non-dysmorphic autism. This suggests that *TMLHE* deficiency may be a risk factor for the development of autism spectrum disorders and that the maintenance of a proper carnitine homeostasis during development may be particularly important in these patients.

Altogether, the results obtained provide new insights on the function and metabolism of carnitine in health and disease. We established the origin and export route of the acylcarnitines that appear in the plasma of patients with mFAO or BCAA oxidation deficiencies and showed that CPT1 does not seem to be crucial for the mitochondrial import of these carnitine derivatives. We have also observed that the carnitine shuttle has a greater contribution to the oxidation of MCFAs than previously expected and that peroxisomes are able to oxidize these fatty acids in cases of mFAO impairment. Moreover, a new inborn error of metabolism is described in the carnitine biosynthesis pathway being its implications in autism spectrum disorders thoroughly discussed.

Keywords: Carnitine shuttle; Mitochondrial fatty acid β-oxidation; Acylcarnitines; Carnitine palmitoyltransferase 1; Carnitine palmitoyltransferase 2; Carnitine/acylcarnitine translocase; Carnitine biosynthesis

Sumário

A carnitina (ou L-carnitina) é um composto crucial no metabolismo energético. No Homem, grande parte da carnitina é proveniente da dieta, nomeadamente através da ingestão de carne e outros produtos de origem animal. No entanto, o organismo tem a capacidade de produzir carnitina endogenamente, um processo do qual indivíduos estritamente vegetarianos dependem para a obtenção de níveis adequados deste composto. A carnitina tem um papel fundamental no transporte de ácidos gordos de cadeia longa do citosol para a mitocôndria, local onde ocorre a β-oxidação.

Apesar da carnitina ser um composto amplamente estudado ao longo dos anos, alguns aspetos relativos ao seu metabolismo e função permanecem pouco claros. Assim, estudos planeados durante esta tese tiveram como objetivos: 1) elucidar a ação do ciclo da carnitina na eliminação de intermediários tóxicos que se acumulam em doenças da β-oxidação mitocondrial dos ácidos gordos e do metabolismo dos aminoácidos de cadeia ramificada, numa função inversa à do seu mecanismo fisiológico; 2) aprofundar o conhecimento acerca do mecanismo de translocação de acilcarnitinas através da membrana externa da mitocôndria; 3) clarificar a contribuição dos peroxissomas para a oxidação de ácidos gordos de cadeia média em casos de deficiência do ciclo da carnitina; 4) proporcionar uma nova perspetiva sobre a importância da carnitina e da sua biossíntese em perturbações do espetro do autismo.

As deficiências na β-oxidação mitocondrial dos ácidos gordos ou no metabolismo dos aminoácidos de cadeia ramificada levam, em geral, à acumulação intramitocondrial de ésteres de acil-coenzima A (acil-CoAs). Estes metabolitos são potencialmente tóxicos e, como tal, são degradados em vias alternativas tais como a ω e ω-1 oxidação, levando à produção de ácidos dicarboxílicos e/ou conjugação com outras moléculas sendo convertidos em acilglicinas ou acilcarnitinas, compostos que podem ser mais facilmente exportados da mitocôndria e da célula. A produção de acilcarnitinas é um dos mecanismos usados para a eliminação mitocondrial e celular dos acil-CoAs que se acumulam devido ao bloqueio enzimático. A especificidade de substrato das duas aciltransferases, tidas geralmente como responsáveis pela síntese mitocondrial de acilcarnitinas, foi extensivamente estudada usando técnicas *in vitro*. Os resultados obtidos demonstraram que as enzimas carnitina palmitoiltransferase 2 (CPT2) e carnitina acetiltransferase são responsáveis pela produção de grande parte das acilcarnitinas encontradas no plasma de doentes com défices ao nível da β-oxidação mitocondrial dos ácidos gordos e do metabolismo dos aminoácidos de cadeia ramificada. Por outro lado, verificou-se que os intermediários *trans-2*-enoil-CoAs não são bons substratos para estas aciltransferases, actuando mesmo como inibidores da CPT2. Esta inibição poderá dever-se a uma interferência destes compostos com o mecanismo catalítico da enzima. Estes dados poderão explicar, não só a ausência de *trans*-2-enoilcarnitinas nos perfis metabólicos em alguns casos de defeitos genéticos ou adquiridos onde se prevê a acumulação dos respetivos ésteres de CoA, mas também a gravidade de

algumas destas patologias, uma vez que os *trans-2*-enoil-CoAs poderão ainda interferir com o metabolismo de outros compostos.

De modo a confirmar os estudos *in vitro* e a clarificar o mecanismo de exportação de acilcarnitinas para o citosol e para o plasma, recorreu-se ao uso de linhas celulares deficientes em proteínas específicas associadas ao transporte de carnitina com e sem silenciamento do gene que codifica para a desidrogenase dos ésteres acil-CoA de cadeia média. Desta forma, foi possível induzir a acumulação intramitocondrial de acil-CoAs, confirmando os resultados obtidos anteriormente para a CPT2. Demonstrou-se que na ausência desta enzima não há produção de acilcarnitinas de cadeia média após a acumulação do respetivo acil-CoA dentro da mitocôndria. Os resultados demonstraram ainda que o transportador carnitina/acilcarnitina translocase (CACT) é responsável pela exportação mitocondrial de acilcarnitinas de cadeia média através da membrana interna da mitocôndria e que a exportação celular destes intermediários não parece depender do transportador de carnitina da membrana plasmática, OCTN2. Do mesmo modo, o mecanismo pelo qual as acilcarnitinas atravessam a membrana externa da mitocôndria permanece por elucidar. Estudos apresentados na literatura sugerem que a enzima carnitina palmitoiltransferase 1 (CPT1) tem a capacidade de se organizar em estruturas oligoméricas que conduzem à formação de complexos hexaméricos na membrana externa da mitocôndria. É ainda sugerido que esta potencial oligomerização da proteína origina uma estrutura semelhante a um poro que poderá facilitar o transporte de acilcarnitinas através da membrana externa da mitocôndria. Os resultados obtidos durante este trabalho revelaram, contudo, que a CPT1 não parece ser crucial na passagem de acilcarnitinas através da membrana externa da mitocôndria para posterior oxidação na matriz mitocondrial ou, pelo menos, esta não parece ser uma via exclusiva para o acesso das acilcarnitinas ao espaço intermembranar.

O papel dos peroxissomas na oxidação de ácidos gordos de cadeia linear média ou longa não é claro. É geralmente aceite que estes substratos são totalmente oxidados na mitocôndria. Em particular, assume-se que os ácidos gordos de cadeia média entram na mitocôndria essencialmente por difusão, ao contrário dos ácidos gordos de cadeia longa que requerem o ciclo da carnitina para aceder à matriz mitocondrial. Os dados obtidos demonstraram que o ácido láurico, normalmente assumido como sendo um ácido gordo de cadeia média, também depende do ciclo da carnitina para ser completamente oxidado na mitocôndria. Adicionalmente foi observado que, em casos de deficiência nas proteínas que compõem o ciclo da carnitina, o ácido láurico é direcionado para os peroxissomas sofrendo pelo menos um ciclo de β-oxidação peroxissomal. Estes resultados sugerem que, ao contrário do que tem sido aceite pela comunidade científica, os peroxissomas têm uma função importante na oxidação de ácidos gordos que não são tipicamente oxidados neste compartimento celular, podendo actuar como um mecanismo compensatório quando a β-oxidação mitocondrial dos ácidos gordos está comprometida.

Por fim, foi demonstrado que a carnitina e a sua biossíntese podem ter um papel relevante em distúrbios neurológicos. Em doentes com autismo não-dismórfico foi encontrada uma deleção no exão 2 do gene *TMLHE*, que codifica para a primeira enzima da biossíntese da carnitina, a dioxigenase da trimetil-lisina. Esta deleção conduz à ausência de proteína, bem como à acumulação de trimetil-lisina e deficiência em γ-butirobetaína no plasma e na urina dos doentes. Deste modo, os resultados obtidos permitiram a caracterização do primeiro erro hereditário da biossíntese da carnitina. Foi ainda observada uma associação desta deficiência genética ao nível do gene *TMLHE* com o autismo não-dismórfico. Estes dados indicam que a deficiência em *TMLHE* pode ser um fator de risco para o desenvolvimento de doenças do espetro do autismo e que a manutenção da homeostase da carnitina durante o desenvolvimento pode ser particularmente importante nestes doentes.

De um modo geral, os resultados obtidos durante este trabalho proporcionam uma nova perspetiva sobre a função e o metabolismo da carnitina em situações fisiológicas ou em casos de doença. Foi possível estabelecer a origem das acilcarnitinas presentes no plasma de doentes com alterações genéticas ou adquiridas da β-oxidação mitocondrial dos ácidos gordos ou do metabolismo dos aminoácidos ramificados e demonstrou-se que a CPT1 não parece ser crucial para a importação mitocondrial destes metabolitos. Observou-se ainda que o ciclo da carnitina apresenta um papel mais importante na oxidação dos ácidos gordos de cadeia média do que até agora assumido e que os peroxissomas podem actuar como um mecanismo compensatório nos casos em que a β-oxidação mitocondrial dos ácidos gordos está comprometida, aceitando como substratos ácidos gordos que não podem ser oxidados na mitocôndria. Por fim, a descoberta de um novo erro hereditário da biossíntese da carnitina permite refletir sobre as suas implicações em perturbações do espetro do autismo.

Palavras chave: Ciclo da carnitina; β-oxidação mitocondrial; Acilcarnitinas; Carnitina palmitoiltransferase 1; Carnitina palmitoiltransferase 2; Carnitina/acilcarnitina translocase; Biossíntese da carnitina

Chapter 1

Aims and outline of the thesis

Carnitine is a crucial compound in fatty acid oxidation and energy metabolism. In the oxidation of fatty acids, especially the long-chain species, carnitine and the carnitine shuttle have a pivotal role in the transport of the esterified acyl units into mitochondria in the form of acylcarnitines. Once in the matrix of this organelle, acylcarnitines are reconverted to acyl-coenzyme A esters (acyl-CoAs), the true substrates of β-oxidation. Although formal proof is lacking, some of the reactions of the carnitine shuttle, mediated by carnitine palmitoyltransferase 1 (CPT1), carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyltransferase 2 (CPT2), are generally assumed to be reversible and, simultaneously, responsible for the export of acylcarnitines to the cytosol. These intermediates are further exported across the cell membrane into the plasma compartment. Presently, plasma acylcarnitine profiling is an essential tool for the early diagnosis of inborn errors of metabolism, namely those affecting fatty acid and branched-chain amino acid oxidation. Nevertheless, the origin of these plasma acylcarnitines has never been thoroughly elucidated. Facing this, the main goals of this thesis were to clarify the mechanisms underlying the mitochondrial synthesis of acylcarnitines and their mitochondrial and cellular export routes, as well as their import into the mitochondrial matrix. Moreover, we aimed to clarify the contribution of the carnitine shuttle and peroxisomes to the oxidation of straight medium-chain fatty acids and additionally, give a new insight on the carnitine biosynthesis pathway.

In **Chapter 2** a detailed review of the state-of-the-art concerning carnitine metabolism and function is presented. This information is complemented by an overview of the mitochondrial and peroxisomal fatty acid β-oxidation and branched-chain amino acid oxidation pathways, as well as the disorders associated. With this background important questions were raised and motivated our studies: What are the enzymes responsible for the synthesis of acylcarnitines and what is their substrate specificity? Is the plasma acylcarnitine profiling found in patients with a fatty acid β-oxidation or branched-chain amino acid oxidation defect a reflection of the intramitochondrial acyl-CoA accumulation which occurs as a result of the blockage upon these pathways? In **Chapters 3 and 4** we aimed to resolve the origin of the acylcarnitines commonly found in these patients. The involvement of CPT2 and carnitine acetyltransferase (CrAT) in this mechanism is generally assumed, but a comprehensive study of the substrate specificity profile of these enzymes has never been performed. With this purpose, detailed *in vitro* studies were performed on the substrate specificity of these key human acyltransferases using homogenates of *Saccharomyces cerevisiae* overexpressing human CPT2 and purified recombinant human CrAT obtained from *Escherichia coli*.

The work reported in **Chapter 5** aimed to confirm the *in vitro* results described in Chapter 3 and to answer the following additional questions: What are the players involved in the mitochondrial and cellular export of acylcarnitines? Is it possible to follow the detoxification path after inducing acyl-CoA accumulation within the mitochondria? To further understand the export route of these acylcarnitines, this chapter describes an

intact cell model developed from patient cell lines deficient in specific proteins potentially involved in the mitochondrial synthesis of carnitine derivatives and in their mitochondrial and cellular export. In these cell lines, a knockdown of the medium-chain acyl-CoA dehydrogenase (MCAD) gene was introduced in order to mimic the intramitochondrial accumulation of medium-chain acyl-CoAs, as commonly observed in MCAD deficiency.

The transport of acylcarnitines into mitochondria across the outer mitochondrial membrane has also remained unclear so far. Carnitine palmitoyltransferase 1 has been proposed to play a role in mediating the transport of long-chain acylcarnitines into the intermembrane space. The work presented in **Chapter 6**, where a patient cell line not expressing CPT1 was used, aimed to clarify whether this enzyme is indeed the exclusive player in the import of acylcarnitines into the mitochondria, or if these metabolites may gain access to the inner mitochondrial membrane transporter CACT through an alternative route, which facilitates the transport of long-chain acylcarnitine derivatives into the matrix.

Contrary to long-chain fatty acids that rely entirely on the carnitine shuttle for their degradation within the mitochondria, it is generally accepted that the oxidation of medium-chain fatty acids is carnitine independent. Moreover, the role of peroxisomes in the oxidation of medium- and long-chain fatty acids has remained elusive. Using as a model cell lines from patients with deficiencies in the members of the carnitine shuttle or in crucial proteins for peroxisome biogenesis, in **Chapter 7** we aimed to understand the contribution of the carnitine shuttle and peroxisomes to the oxidation of medium-chain fatty acids.

In **Chapter 8** some light was shed on the importance of the biosynthesis of the carnitine moiety itself. In fact, in situations such as starvation and vegetarian diet, the body totally depends on the adequate function of the proteins involved in the carnitine biosynthetic pathway. Deficiencies in the enzymes contributing to the endogenous formation of carnitine have never been reported and thus, the implications of an impaired carnitine biosynthesis are so far unknown. In this chapter, a novel inborn error of metabolism affecting the biosynthesis of carnitine is described in children with non-dysmorphic autism. Making use of genetic and biochemical studies the work reported in Chapter 8 aimed primarily to find a correlation between this deficiency in the carnitine metabolism and autism spectrum disorders and ultimately to discuss the potential role of carnitine in the mechanisms of neurologic disease.

The final chapter comprises the concluding remarks of the thesis where the results obtained during its development and their impact in carnitine metabolism and function are discussed. **Chapter 9** also includes a reflection on the aspects that still demand further attention and some perspectives for future work.

Chapter 2

General introduction

1. Carnitine function

Carnitine (L-3-hydroxy-4-*N,N,N*-trimethylamino-butyrate; L-carnitine) is a quaternary ammonium compound crucial in energy metabolism, described as a conditionally essential nutrient [1, 2]. It has a primary role in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place [3]. Carnitine is also used in the transfer of peroxisomal β-oxidation intermediates and products to mitochondria to be completely metabolized through β-oxidation and/or the Krebs cycle [4, 5]; in the modulation of the intramitochondrial acyl-CoA/CoA ratio [6, 7]; and as a detoxifying agent facilitating the elimination from the cell of accumulating acyl units in the form of carnitine esters [8]. In humans, about 75% of carnitine is obtained from the diet. L-carnitine, the biologically active stereoisomer, is found essentially in meat and other animal products being very limited in plants [2, 9]. Therefore, in strictly vegetarian individuals, the amount of carnitine derived from the diet is quite low and the body mostly relies on its endogenous synthesis.

1.1 Carnitine biosynthesis

The biosynthesis of L-carnitine occurs in kidney, liver and brain from the amino acids lysine and methionine [9]. The carbon backbone of carnitine is provided by lysine while methionine is the donor of the 4-*N*-methyl groups [10-12]. The N-methylation of lysine residues on the 6-amino group occurs as a post-translational event in certain proteins [13, 14]. This reaction is catalyzed by specific methyl-transferases that use S-adenosylmethionine as the methyl donor to form 6-*N*-trimethyllysine (TML) residues [15]. Protein hydrolysis in lysosomes results in the release of TML, the first metabolite in the biosynthesis of carnitine [16].

After lysosomal degradation, TML residues undergo four enzymatic reactions before the formation of L-carnitine (Fig. 1). The first reaction corresponds to the hydroxylation of TML on the third position by the enzyme trimethyllysine dioxygenase (TMLD), encoded by the *TMLHE* (trimethyllysine hydroxylase, epsilon) gene, which maps to the long arm of the X chromosome. The resultant 3-hydroxy-6-*N*-trimethyllysine (HTML) undergoes an aldolytic cleavage to yield 4-trimethylaminobutyraldeyde (TMABA) and glycine in a reaction catalyzed by HTML aldolase (HTMLA). TMABA is oxidized by TMABA dehydrogenase (TMABA-DH) which results in the formation of 4-*N*-trimethylaminobutyrate (γ-butyrobetaine; γ-BB). Finally, γ-BB is hydroxylated to L-carnitine by the enzyme γ-butyrobetaine dioxygenase (γ-BBD) [16].

1.2 Carnitine homeostasis

In humans, exogenous TML does not function efficiently as a precursor for carnitine biosynthesis. Loading tests have shown that after ingestion of TML, most of the compound (approximately 75%) is excreted unchanged in the urine [9, 17]. Tissues such as the heart and muscle are not able to efficiently absorb TML from the circulation and it is believed that the TML produced intracellularly is converted to γ-BB in the tissue of origin [9]. The γ-BB produced is then secreted into circulation and readily absorbed by the tissues that contain γ-BBD (liver, kidney and brain) [18], where final conversion to carnitine takes place. The carnitine produced is further transported through the blood into the

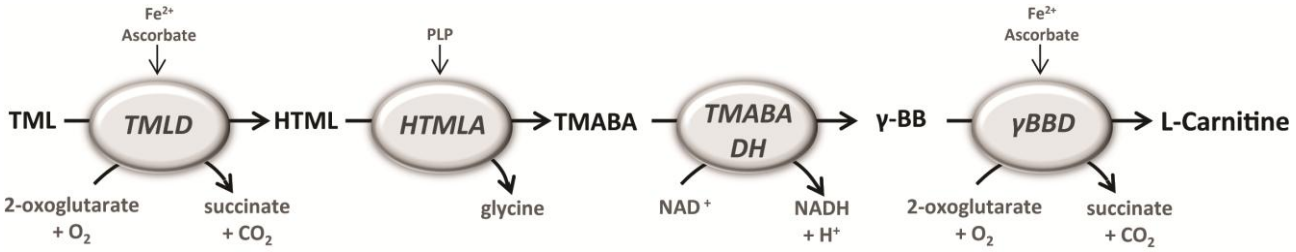

Figure 1 – Schematic representation of the carnitine biosynthesis pathway. 6-*N*-trimethyllysine (TML) residues are hydroxylated by TML-dioxygenase (TMLD) to 3-hydroxy-TML (HTML). HTML is converted to 4-trimethylaminobutyraldeyde (TMABA) by HTML aldolase (HTMLA), which uses pyridoxal 5'-phosphate (PLP) as cofactor. TMABA-dehydrogenase (TMABA-DH) oxidizes TMABA to 4-*N*-trimethylaminobutyrate (γ-butyrobetaine; γ-BB), which is hydroxylated to carnitine by γ-BB-dioxygenase (γ-BBD).

tissues where it is needed for fatty acid oxidation. Its cellular uptake is mediated by the plasmalemmal high affinity organic cation/carnitine transporter (OCTN2) and other low affinity transporters present in the plasma membrane.

Carnitine homeostasis is maintained by a modest rate of endogenous synthesis, absorption from dietary sources, and efficient tubular reabsorption by the kidney. However, urinary carnitine excretion largely depends on the diet and the kidney has been shown to adapt to a higher carnitine intake by reducing the efficiency of carnitine reabsorption [19, 20].

1.3 The cellular uptake of carnitine: the organic cation/carnitine transporter

The plasmalemmal organic cation/carnitine transporter – OCTN2 – is a member of the solute carrier family 22 of sodium-dependent organic cation/carnitine transporters (SLC22A5). The *SLC22A5* gene, which encodes in humans the OCTN2 transporter, is mapped to chromosome 5 (5q31) [21, 22]. OCTN2 transports carnitine with high affinity in a sodium-dependent manner [22, 23]. This transporter is also responsible for the sodium-independent transport of other organic cations [24, 25]. Human OCTN2 contains 557 amino acids organized in 12 putative transmembrane domains with the N- and C-termini on the cytoplasmic side [21]. In humans, OCTN2 is expressed in several tissues including placenta, skeletal muscle, heart and kidney. In kidney, it is localized on the apical membrane of renal tubular epithelial cells, which demonstrates the importance of OCTN2 for reabsorption of carnitine after glomerular filtration [26]. OCTN2 has a marked homology to the low affinity sodium-independent carnitine transporter OCTN1, as well as with OCTN3 which is only known from rat and mouse [22, 27]. The OCTN2 transporter presents a higher affinity for carnitine and short-chain acylcarnitines as substrates but it is also efficient in facilitating the cellular uptake of betaine [28, 29]. The nuclear receptor peroxisome proliferator activated receptor alpha (PPARα) regulates the expression of several genes involved in metabolism and has been described to up-regulate as well the usually low OCTN2 expression in mouse liver, leading to an increase in the hepatic transport of carnitine and of its precursor γ-BB [30, 31].

1.4 The mitochondrial uptake of carnitine: the carnitine/acylcarnitine transporter

The carnitine/acylcarnitine translocase (CACT) is an inner mitochondrial membrane (IMM) transporter, mediating the electroneutral exchange between acylcarnitine esters and free carnitine [32-34], as well as the unidirectional transport of free carnitine into the mitochondrial matrix [35]. This transporter, with 301 amino acid residues, is a member of the solute carrier family 25 (SLC25A20) and the human gene, cloned and sequenced by Huizing *et al* [36] is located on chromosome 3 (3p21.31) [37]. Expression of CACT in mouse liver is up-regulated by PPARα and PPARδ [38]. Similarly to other mitochondrial carriers, CACT displays three repeated homologous regions, being its monomer likely organized into six transmembrane alpha-helixes linked asymmetrically by five hydrophilic loops with the N-terminus, the C-terminus and two small loops facing the intermembrane space and three extensive loops exposed to the matrix side [39-41]. Functional properties of CACT have been investigated in isolated rat liver mitochondria after purification from living tissues [34, 42] or as a recombinant protein reconstituted in liposomes [43-45]. Both rat and human transporters accept acylcarnitines from 2 to 18 carbon atoms with higher affinity for the long-chain species [42-45]. Differences in the *K*m values on the cytosolic and on the matrix side imply that the preferential reaction is the transport of acylcarnitines from the intermembrane space into the matrix [46, 47]. Despite the information gathered towards the rat CACT, knowledge on the structure and the kinetic properties of the human homologue is still limited [44, 45].

1.5 The carnitine acyltransferases

Carnitine acyltransferases are a group of enzymes that catalyze the transfer of acyl groups between coenzyme A (CoA) and carnitine, being crucial in the metabolism of fatty acids. The carnitine acyltransferases' family includes four members: carnitine acetyltransferase, carnitine octanoyltransferase, carnitine palmitoyltransferase 1 and carnitine palmitoyltransferase 2.

1.5.1 The carnitine acetyltransferase

Carnitine acetyltransferase (CrAT) is a soluble enzyme present in peroxisomes, mitochondria and in the endoplasmic reticulum [7, 48, 49]. It is active with acyl groups with a chain-length of 2 to 10 carbon atoms [50-53]. The mitochondrial and peroxisomal enzymes contain about 600 amino acids and differ only in the initial mitochondrial targeting signal [54]. CrAT is widely distributed in different tissues [55, 56] and the corresponding gene (*CRAT*) is localized to the chromosome 9 (9q34.1) [57]. In mitochondria, CrAT has an important role in the maintenance of the acyl-CoA/CoA pool [7, 8] and in the regulation of the mitochondrial energetic status [56]. The production of acetylcarnitine by CrAT has been associated with positive effects in several models of neuronal dysfunction [58-62], namely through the redirection of excess carbon equivalents to tissues with less ability to use long-chain fatty acids (such as the brain) [56]. Furthermore, it has been recently described that CrAT has an impact in whole body glucose homeostasis due to its role in allowing tissue efflux of acetylcarnitine derived from the pyruvate dehydrogenase complex (PDHc) in a mouse muscle-specific CrAT knockout model [55]. The structural studies performed with the human CrAT and the resolution of the 3D structure of this enzyme were major advances in the understanding of the catalytic mechanism of acyltransferases as a whole [54, 63].

1.5.2 The carnitine octanoyltransferase

Carnitine octanoyltransferase (CrOT) is found specifically in the peroxisome [64, 65] and this 612 amino acid protein is encoded by the gene *CROT,* which has been localized to the chromosome 7 (7q21.1) [66]. It has preference for substrates with medium-chain length (between 6 and 10 carbon atoms) but it also handles long-chain acyl-CoAs, although to a lesser extent [51]. Very long-chain, branched-chain and long-chain dicarboxylic fatty acids undergo β-oxidation in the peroxisomes. The medium-chain acyl-CoAs that result from this oxidation are converted to carnitine esters by CrOT and exported to the cytosol. These intermediates are further imported into the mitochondria for complete oxidation [7]. CrOT has 36% amino acid sequence identity with CrAT, and like CrAT its crystal structure has been determined [67]. Significant differences are observed in the acyl-CoA binding site of these two enzymes, which in CrOT is larger and thus more suited for medium-chain acyl groups [67]. CrOT has been implicated in the regulation of ketogenesis due to its role in the transfer of shortened fatty acids from peroxisomes to mitochondria [68]. Furthermore, it has been recently suggested that CrOT may regulate very long-chain fatty acid metabolism through alteration of the amount of medium-chain fatty acids produced in the peroxisome [69].

1.5.3 The carnitine palmitoyltransferase 1

Integrated in the outer mitochondrial membrane (OMM), carnitine palmitoyltransferase 1 (CPT1) catalyzes the transesterification reaction between long-chain acyl-CoA and acylcarnitine esters. Three isoforms are known to date: CPT1A (773 amino acids) expressed ubiquitously with high levels in liver and kidney, CPT1B (772 amino acids) mainly present in cardiac and skeletal muscle, and CPT1C (798 amino acids) expressed in brain [7, 70]. These isoforms are encoded by different genes localized on the chromosome 11 (11q13), 22 (22qter) and 19 (19q13), respectively for CPT1A, CPT1B and CPT1C [70, 71]. The reaction catalyzed by CPT1 is considered the rate limiting step of mitochondrial fatty acid β-oxidation and its activity is strongly regulated by malonyl-CoA [72], the first intermediate of the fatty acid biosynthesis. CPT1 has two transmembrane domains connected by a loop protruding to the intermembrane space (IMS) and both N- and C-termini appear to be projected to the cytosol [73], although this remains a matter of discussion [74]. Interactions between the N- and the C-terminus help to stabilize the catalytic site [74, 75] and seem to modulate malonyl-CoA sensitivity, even though the mechanism is still poorly understood [76-78].

It has been suggested that the transmembrane domain 2 of CPT1A is responsible for the oligomerization of the enzyme, which would result in the formation of hexameric complexes potentially arranged as a pore within the OMM [79, 80]. Moreover, CPT1A has recently been implicated in the formation of hetero-oligomeric complexes in the OMM with long-chain acyl-CoA synthetase (ACSL) and the voltage dependent anion channel

(VDAC) [74]. However, the functional relevance of such complexes remains unknown.

1.5.4 The carnitine palmitoyltransferase 2

Carnitine palmitoyltransferase 2 (CPT2) contains 658 amino acid residues and is associated with the inner face of the IMM having only one isoform, ubiquitously expressed [7]. The gene encoding this homotetrameric protein locates in humans on chromosome 1 (1p32) [81]. This enzyme is malonyl-CoA insensitive and its main function is to catalyze the conversion of long-chain acylcarnitines into the respective acyl-CoA esters in the presence of CoA, providing the substrates for β-oxidation. Studies on the crystal structure of rat CPT2 have shown an insert that serves as a membrane anchor for the enzyme [82]. It is also suggested that this insert could physically interact with CACT, allowing the direct channeling of acylcarnitine substrates to the active site of CPT2 [82-84]. Studies on human CPT2 show higher activity towards medium and long-chain substrates, its specificity ranging from C8 to C20 carbon chain [85]. Furthermore, CPT2 has been

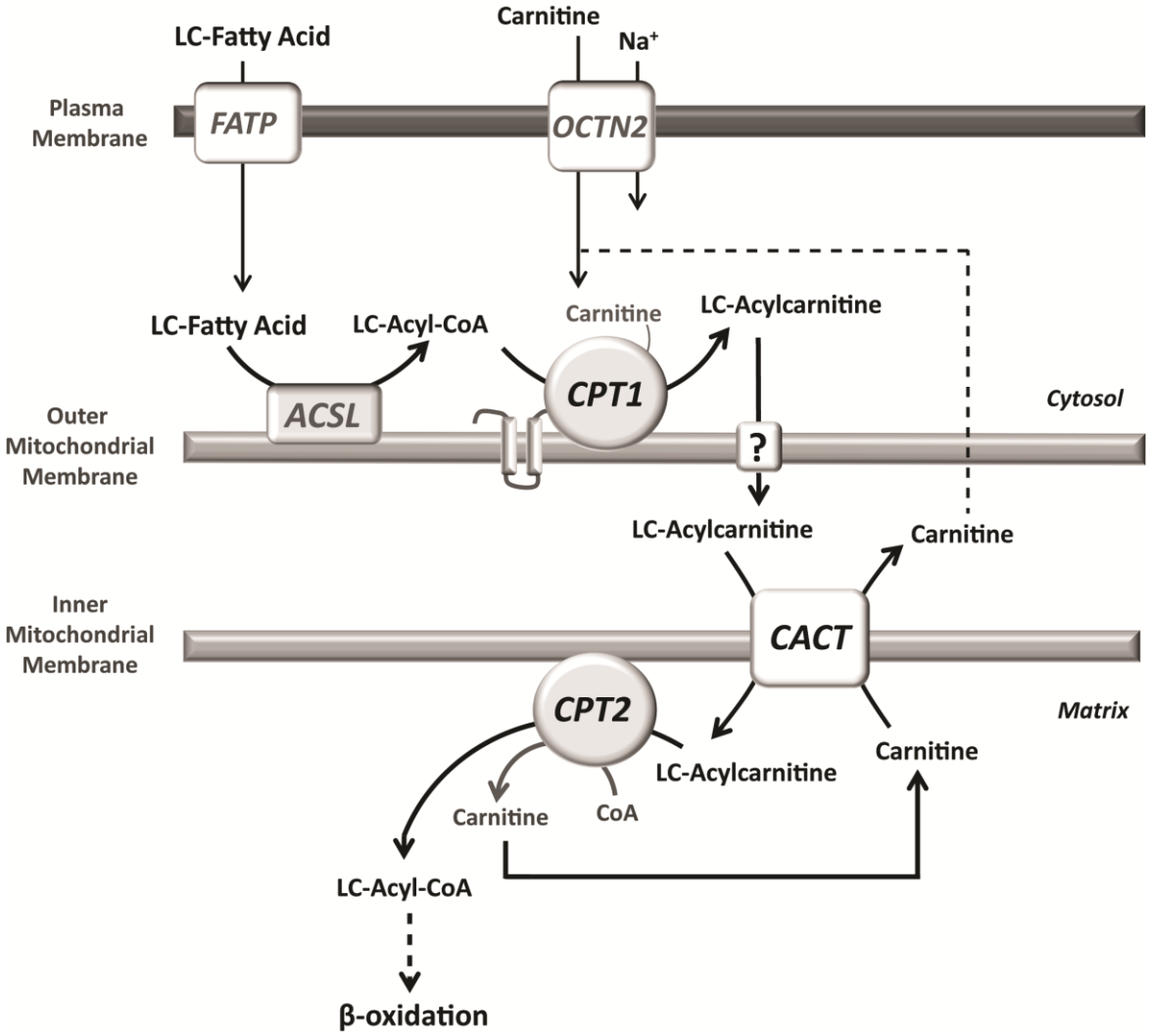

Figure 2 - Schematic representation of the carnitine shuttle. Long-chain fatty acids (LCFA) enter the cell via various plasmalemmal transporters namely the fatty acid transport protein (FATP), being activated to coenzyme A (CoA) esters in the cytosol by the long-chain acyl-CoA synthetase (ACSL). Carnitine palmitoyltransferase 1 (CPT1) converts the long-chain (LC) acyl-CoAs into the respective acylcarnitines, which are transported into the mitochondrial matrix by the carnitine/acylcarnitine translocase (CACT) in exchange with free carnitine. In the matrix, carnitine palmitoyltransferase 2 (CPT2) reconverts the LC-acylcarnitines into acyl-CoA esters, the true substrates for β-oxidation.

described to reverse its physiological mechanism *in vitro* and accept acyl-CoA esters as substrates converting them into the corresponding acylcarnitines [85, 86]. Pharmacological up-regulation of CPT2 might be achieved through the action of PPAR agonists such as bezafibrates [87]. These compounds seem to enhance *CPT2* gene expression and CPT2 residual activity, restoring long-chain fatty acid oxidation capacities in mild cases of CPT2 deficiency [88].

1.6 The carnitine shuttle and regulation of carnitine transfer

In order to be metabolized in the mitochondria, long-chain fatty acids must undergo activation by long-chain acyl-CoA synthetases prior to its transport into the mitochondrial matrix [89-91]. Transport of activated long-chain fatty acids (long-chain acyl-CoAs) into the mitochondrial matrix is carried out by the carnitine shuttle (Fig. 2). This system requires the presence of L-carnitine and is composed of the acyltransferases CPT1 and CPT2 as well as the mitochondrial carrier CACT. Long-chain acyl-CoAs are firstly converted into the corresponding carnitine esters by CPT1. The resulting acylcarnitines are then transported across the IMM by CACT, in exchange with free carnitine. The final step of this cycle, catalyzed by CPT2, reconverts the acylcarnitines into the respective acyl-CoA esters that can then undergo β-oxidation. Carnitine returns to the cytosol as free carnitine for another cycle or it can be exported out of the cell in the form of acylcarnitines [3], potentially through the reversible activity of CPT2 and CACT.

2. Mitochondrial fatty acid β-oxidation

Once in the mitochondria, acyl-CoA esters enter the β-oxidation pathway (mFAO), a four-step mechanism where the β-carbon of the acyl unit is oxidized, being the acyl-CoA shortened in two-carbon atoms which are released as acetyl-CoA. Each of these steps is catalyzed by chain-length specific enzymes [3] (Fig. 3). In the first step the acyl-CoA is dehydrogenated, yielding a *trans*-2-enoyl-CoA intermediate. Depending on the length of the carbon chain, this reaction is catalyzed by specific acyl-CoA dehydrogenases: short- (SCAD for C4-C6 acyl-CoAs), medium- (MCAD for C4-C12 acyl-CoAs), long- (LCAD for C8-C20 acyl-CoAs) or very long-chain acyl-CoA dehydrogenase (VLCAD for C12-C24 acyl-CoAs). However, the exact role of LCAD in mitochondrial β-oxidation is still quite unclear. It has been shown that this enzyme is not only capable of handling straight-chain fatty acids, but also branched-chain substrates such as 2,6-dimethylheptanoyl-CoA [92] and several un-saturated fatty acids [93, 94]. All acyl-CoA dehydrogenases are localized in the mitochondrial matrix, with exception of VLCAD which is loosely bound to the inner mitochondrial membrane. VLCAD is also distinguishable from the other enzymes in that it is a dimer, whereas the other acyl-CoA dehydrogenases are homotetramers [95]. Each subunit contains one molecule of non-covalently bound flavin adenine dinucleotide (FAD) which accepts the electrons involved in the dehydrogenation reaction and is further reoxidized by the electron-transfer flavoprotein (ETF), via which these electrons are transferred to the respiratory chain [96, 97]. The second step of the β-oxidation pathway is the hydratation of the *trans*-2 double bond of the enoyl-CoA intermediate, leading to the formation of a 3-hydroxyacyl-CoA. This step is catalyzed by two different enoyl-CoA hydratases: short-chain enoyl-CoA hydratase (crotonase) and long-chain enoyl-CoA hydratase (LCEH). Crotonase is active with fatty acyl units of chain-length up to 10 carbons and LCEH handles the long-chain enoyl-CoA intermediates [98]. LCEH is part of the mitochondrial trifunctional protein (MTP), a heterooctamer associated with the IMM also carrying long-chain 3-hydroxyacyl-CoA and 3-ketoacyl-CoA thiolase activities [98, 99]. A second dehydrogenation step is catalyzed by two different NAD^+-dependent enzymes with 3-hydroxyacyl-CoA dehydrogenase activity (specific for short- and long-chain substrates) yielding 3-ketoacyl-CoA intermediates and NADH, the latter being further reoxidised by the complex I of the respiratory chain [100]. Short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) has broad substrate specificity (C4 to C16) but shows optimal activity with C6 substrates. The other enzyme, long-chain 3-hydroyacyl-CoA dehydrogenase (LCHAD), also part of the MTP complex, has higher activity towards long-chain intermediates [101]. In the final step of the β-oxidation cycle the 3-ketoacyl-CoA undergoes a thiolytic cleavage catalyzed by short- (SKAT),

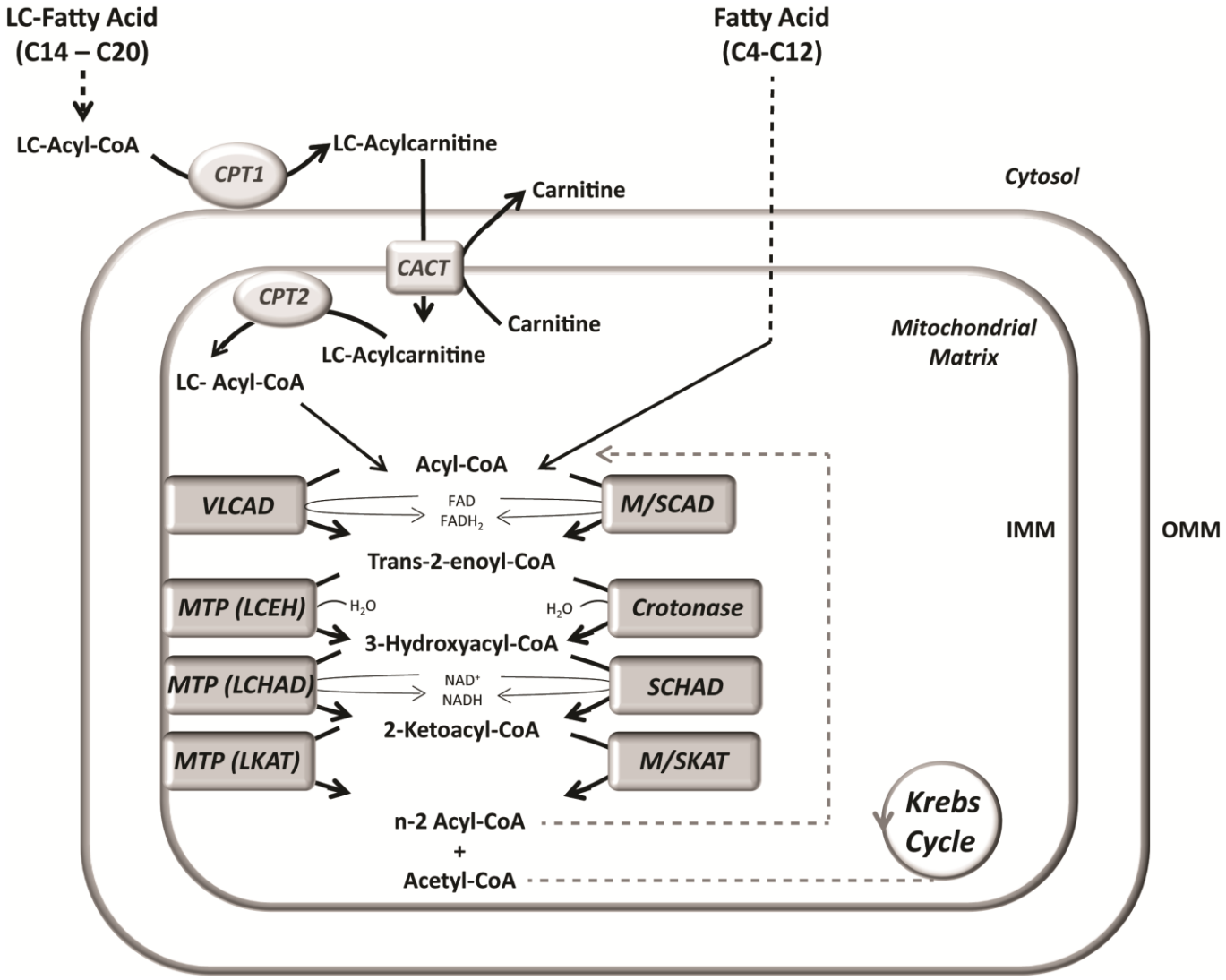

Figure 3 - Schematic representation of the mitochondrial fatty acid β-oxidation. The first step of the β-oxidation pathway is catalyzed by specific acyl-CoA dehydrogenases, depending on the chain-length of the substrate: very long-chain acyl-CoA dehydrogenase (VLCAD) for long-chain (LC) substrates and medium- or short-chain acyl-CoA dehydrogenase (M/SCAD), for medium- and short-chain acyl-CoA esters. The next reaction is catalyzed by long-chain enoyl-CoA hydratase (LCEH) or short-chain enoyl-CoA hydratase (crotonase) for long- and short-chain substrates, respectively. LCEH is part of the mitochondrial trifunctional protein (MTP), a heterooctamer associated with the inner mitochondrial membrane (IMM also including long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and long-chain 3-ketoacyl-CoA thiolase (LKAT) activities. The third reaction is catalyzed by LCHAD or short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD). Finally, an acyl-CoA with the chain shortened by 2 carbon atoms and acetyl-CoA are produced by either long-, medium- or, short-chain 3-ketoacyl-CoA thiolase (LKAT, MKAT and SKAT, respectively) and the new acyl-CoA can enter another cycle of β-oxidation. FAD, flavine adenine dinucleotide (oxidized); $FADH_2$, flavine adenine dinucleotide (reduced); NAD^+, nicotinamide adenine dinucleotide (oxidized); NADH, nicotinamide adenine dinucleotide (reduced).

medium- (MKAT) or long-chain 3-ketoacyl-CoA thiolase (LKAT, which is also part of MTP), forming an acyl-CoA, with the chain shortened by two carbon atoms, and acetyl-CoA. The resultant acyl-CoA will undergo a new cycle of β-oxidation and the acetyl-CoA can either enter the Krebs cycle or be used to produce ketone bodies in the liver or kidney [100].

2.1 Mitochondrial fatty acid β-oxidation disorders

Mitochondrial fatty acid β-oxidation disorders (mFAOD) are a clinically and biochemically heterogeneous group of rare inherited metabolic diseases, with an incidence of approximately 1:10,000 to 1:50,000 births [102, 103]. Defects in one or more enzymes of the β-oxidation pathway frequently lead to severe deficiencies. The first

inborn error of long-chain fatty acid oxidation reported was CPT2 deficiency and only about ten years later, in 1982, MCAD deficiency was identified [104]. Since then, inherited defects of several enzymes directly involved in this process have been identified in humans. Affected patients usually present hypoketotic hypoglycemia with hepatic, cardiac and/or muscular symptoms. In specific cases, such as MTP and CPT2 deficiencies, patients might also develop peripheral neuropathy. Biochemical manifestations of these disorders comprise deficient production of acetyl-CoA and ketone bodies, and consequently an energy deficit, and accumulation of free fatty acids, acyl-CoA and acylcarnitine intermediates upstream of the enzymatic block [3], which contribute to the pathogenesis of the various mitochondrial fatty acid β-oxidation disorders.

2.1.1 Defects of fatty acid transport

These disorders include primary systemic carnitine deficiency, CPT1, CPT2 and CACT deficiencies.

Primary systemic carnitine deficiency: This pathology has been associated with a defect at the level of the plasma membrane carnitine transporter OCTN2 [105]. Symptoms usually present in infancy and include muscle weakness and cardiomyopathy. This deficiency is characterized by low total free carnitine in plasma, typically less than 5 µmol/L (normal values range from 25 to 50 µmol/L) [106]. The frequency reported is approximately 1:50,000, with about 1% of the population being a carrier for this disease [106, 107]. Treatment with oral carnitine (starting at 50 mg/kg/day) reverses the symptoms and prevents acute crises and cardiomyopathy [106, 108].

CPT1 deficiency: Deficiency at the level of CPT1 generally presents between birth and 18 months of age with altered mental status and low levels of long-chain acylcarnitines. Biochemical findings include non-ketotic hypoglycemia, mild hyperammonemia, elevated liver function tests and elevated free fatty acids, among others. Furthermore, in contrast with other mFAOD, CPT1 deficiency is consistently associated with elevated plasma carnitine levels [109]. This is a rare disorder with only about 30 cases known to date [110, 111], which is consistent with the crucial role of the CPT1 enzyme in fatty acid metabolism. Despite the small number of cases reported, several mutations have been identified in CPT1 deficient patients (for review see [112]), some of which show a fair correlation between the severity of the enzymatic impairment caused by the mutation and the clinical presentation [113]. However, only the deficiency of the liver type (CPT1A) has been reported in humans, likely because the importance of CPT1 in heart and brain might turn deficiencies in the CPT1B and CPT1C isoforms incompatible with life. Treatment includes the maintenance of high glucose levels in plasma through glucose infusions during the neonatal period and in acute metabolic crisis, as well as a medium-chain triglycerides (MCT) enriched diet [109, 112].

CPT2 deficiency: This is one of the most common inherited disorders of fatty acid metabolism [114, 115]. Three clinical phenotypes can be distinguished including neonatal, infantile and adult presentations. The adult form, firstly identified in 1973 [116], is the most common and usually presents in young adults with recurrent myoglobinuria. The infantile form generally presents in early childhood with fasting-induced hypoketotic hypoglycemia, liver failure, cardiomyopathy, and peripheral neuropathy. CPT2 deficiency is treatable if diagnosed early, but otherwise it is potentially fatal. The perinatal form is the least common clinical presentation of CPT2 deficiency and is usually fatal [115, 117]. Elevated long-chain acylcarnitine species and low carnitine levels are observed. Current treatment involves dietary fat restriction and increased intake of carbohydrates [118]. Pharmacological treatment with bezafibrates in patients with the muscular form of CPT2 deficiency has proved beneficial with significant improvements in exercice tolerance [88].

CACT deficiency: Deficiency of the mitochondrial carnitine transporter CACT is a severe and very rare disorder characterized by the accumulation of long-chain acylcarnitine esters (identical to CPT2 deficiency) and severe secondary carnitine deficiency. These acylcarnitine intermediates are the most relevant findings in plasma and bile of suspected patients. Severe cardiomyopathy, muscle weakness and hypoketotic hypoglycemia are the most common symptoms [119]. When diagnosis is

performed at an early stage the chances of a gradual improvement are higher. Nevertheless, these patients are extremely vulnerable and the long-term prognosis is not always encouraging. Avoidance of fasting, maintenance of a high carbohydrate intake during illness and ammonia detoxification help in the management of acute episodes [41, 119].

2.1.2 Defects of the β-oxidation enzymes

Short-chain acyl-CoA dehydrogenase deficiency (SCAD): The first case of SCAD deficiency was reported in 1984 [120] and since then an increasing number of cases have been described. Even though, the clinical phenotype remains uncertain to this day. Neonatal features may include feeding difficulties, hypotonia, lethargy, hypoglycemia, and brain malformations [121]. Biochemical findings include elevated levels of butyrylcarnitine and urinary excretion of ethylmalonic acid. The clinical heterogeneity of this deficiency makes its characterization still rather unclear and difficult. Potential treatment includes riboflavin, the precursor of the SCAD co-factor (FAD) [122]. In addition, it is generally believed that patients should avoid fasting although there are currently no studies to support this assumption.

Medium-chain acyl-CoA dehydrogenase deficiency (MCAD): MCAD deficiency is the most common defect of the β-oxidation pathway [123]. Non-ketotic hypoglycemia, abnormal urinary and plasma metabolites, C6-C10 dicarboxylic acids and medium-chain acylcarnitines, are biochemical findings usually triggered by prolonged fasting, intercurrent infection and intense physical exercise. Nevertheless, many patients may remain asymptomatic through life being only diagnosed subsequently to family studies. With treatment consisting of fasting avoidance, dietary restriction of fat, and L-carnitine therapy, morbidity and mortality can be prevented [123].

Very long-chain acyl-CoA dehydrogenase deficiency (VLCAD): The first cases reported with VLCAD deficiency were in fact described as LCAD deficient patients because the VLCAD enzyme had not yet been discovered [124]. Only later VLCAD deficiency was correctly recognized and characterized [125, 126], being the most common mitochondrial long-chain fatty acid oxidation defect with 1:50,000 to 1:100,000 individuals affected [127]. This pathology has a severe phenotype characterized by early onset, high mortality rate and cardiomyopathy in most of the patients [128]. A milder phenotype is also described showing a delayed disease onset, lower mortality rate, hypoketotic hypoglycemia and absence of cardiomyopathy [129]. Avoidance of fasting, reduction of long-chain fat intake and supplementation with MCT generally avoids metabolic crisis and long-term recovery can be successful.

Short-chain 3-hydroxyacyl-CoA dehydrogenase deficiency (SCHAD): SCHAD catalyses the penultimate step in β-oxidation of short-chain fatty acids. SCHAD deficiency is a rare disorder with less than 20 cases described and is characterized by an urinary organic acid profile with elevated medium-chain dicarboxylic and 3-hydroxydicarboxylic metabolites and lowered enzyme activity [130, 131]. Patients present hyperinsulinemia in association with hypoglycemia [132-136] and the mechanism of hyperinsulinemia has been defined as a regulatory effect of the SCHAD on glutamate dehydrogenase, independent from the enzyme activity [137]. Treatment includes frequent feeding and therapy with diazoxide, which targets the ATP-sensitive potassium channel in the pancreatic β-cell [138, 139].

Isolated long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD) and mitochondrial trifunctional protein (MTP) deficiencies: LCHAD is part of the MTP protein complex associated with the inner face of the IMM. Besides LCHAD, MTP also carries LCEH and LKAT activities. Thus, MTP deficiency can be classified into two categories: isolated LCHAD deficiency and complete MTP deficiency with the compromise of all the three enzyme activities [140]. Most commonly, patients have isolated LCHAD deficiency [141]. The distinction between LCHAD and MTP deficiencies is not clear, and also the acylcarnitine profiles are identical [140]. Symptoms associated with deficiency in MTP/LCHAD include hypoglycemia, cardiomyopathy, acute liver dysfunction and sudden death [142, 143]. In addition, progressive

peripheral neuropathy and pigmentary retinopathy are also observed, which among β-oxidation pathologies seem to be unique features of MTP and LCHAD deficiencies [143, 144]. Moreover, in addition to the ophthalmologic manifestations mentioned above, serious pregnancy complications such as HELLP (Hemolysis, Elevated Liver enzymes, Low Platelets) syndrome and AFLP (Acute Fatty Liver of Pregnancy) have been associated with carriers of LCHAD deficiency [145, 146]. Treatment of these deficiencies, like for the other long-chain fatty acid oxidation disorders, generally consists of avoidance of fasting with frequent meals and MCT supplementation to prevent activation of long-chain fatty acid oxidation [147, 148]. Carnitine supplementation is also often recommended but its benefit/risk ratio is often debated as it might lead to the accumulation of likely deleterious long-chain acylcarnitines [143]. Although treatment may improve long-term prognosis in many cases, it is not sufficient to prevent the progression of the disease.

2.2 Pathogenesis of mitochondrial long-chain fatty acid β-oxidation

Deficiencies affecting enzymes specific for long-chain fatty acyl substrates generally lead to more severe phenotypes than the ones with specificity for short- and medium-chain substrates. Pathologies concerning long-chain fatty acid oxidation are complex and clinically heterogeneous. Additionally to the symptoms usually observed in mFAOD, defects affecting long-chain mFAO tend to be more severe and present unusual features. Such complications may be associated with accumulation of toxic intermediates from anomalous β-oxidation of long-chain fatty acids [143]. As described above, deficiencies in the mFAO pathway result in the intramitochondrial accumulation of acyl-CoA metabolites. Acyl-CoA esters and particularly long-chain fatty acyl-CoAs and their β-oxidation intermediates have been shown to be potent inhibitors of several enzymes and transport systems namely ATP synthase, ATP/ADP carrier and the dicarboxylate carrier [149-152]. An inhibitory effect upon these proteins will contribute to further compromise energy metabolism including the normal function of the respiratory chain, responsible for the cellular reoxidation of the reducing equivalents $FADH_2$ and NADH formed during mFAO. Furthermore, as a consequence of the accumulation of acyl-CoA intermediates, CoA depletion is also observed. Altogether, it is expected to occur an elevation of the acyl-CoA/CoA, ADP/ATP and $NADH/NAD^+$ ratios, essential factors in the regulation of certain enzymes, particularly the pyruvate dehydrogenase complex (PDHc). When elevated, these ratios will activate the pyruvate dehydrogenase kinase (PDK) leading to an inactivation of the PDHc by phosphorylation and consequent elevation of pyruvate and lactate [153, 154]. In fact, elevated lactate is one of the frequent findings in long-chain mFAOD such as MTP/LCHAD and VLCAD deficiencies (both *in vivo* and *in vitro*) [143, 147], which may contribute to the severity of these disorders. Nevertheless, high lactate levels are accompanied by elevated lactate/pyruvate (L/P) ratio in MTP/LCHAD deficiency but not in VLCAD deficiency where L/P ratios are within the normal range [155]. This fact may explain the apparent specificity of the signs and symptoms of MTP/LCHAD deficient patients, mentioned above when compared with other similarly severe long-chain mFAODs. Furthermore, it may also suggest a differential effect of the intermediates that specifically accumulate in MTP/LCHAD deficiencies (*trans*-2-enoyl-CoA, 3-OH-acyl-CoA and respective acylcarnitines) upon the enzymes and transport systems involved in energy metabolism, namely pyruvate transport and/or PDHc. Long-chain acylcarnitines have been also recognized as potentially deleterious for the cells and ischemic damage, arrhythmias, and impaired postischemic cardiac function have been associated with high tissue levels of these intermediates [156, 157]. However, it has been also proposed that these ischemic changes result from the inhibition of long-chain fatty acid β-oxidation and reduced energy production, and not from the accumulation of tissue acylcarnitines [158]. Therefore, as mentioned above, the benefit of carnitine supplementation in patients with defects of long-chain mFAO is still controversial, as it may induce the production of potentially toxic acylcarnitine species. In fact, *in vivo* studies with the VLCAD knockout mouse model show increased acylcarnitine levels induced by carnitine supplementation, as well as *in vitro* cytotoxic effects of palmitoylcarnitine in hepatic cells [159]. Although the toxicity of the

accumulating acylcarnitines and acyl-CoAs in MTP/LCHAD deficiency has been also suggested to play a role in the development of the retinopathy and neuropathy characteristically observed in these patients, formal proof is still lacking [143, 160].

3. Peroxisomal fatty acid β-oxidation

Peroxisomes are essential organelles with important functions in lipid metabolism. They are responsible for the α-oxidation of 3-methyl branched-chain fatty acids and β-oxidation of other lipids, including long and very long-chain fatty acids, long-chain dicarboxylic fatty acids, 2-methyl-branched fatty acids (like pristanic acid), di- and trihydroxycholestanoic acid (DHCA and THCA), eicosanoids and xenobiotic carboxylic acids [161, 162]. However, while mitochondrial β-oxidation ultimately leads to the production of acetyl-CoA (and therefore of energy), peroxisomal β-oxidation is an incomplete process. Fatty acids that are poorly metabolized in the mitochondria are activated to the corresponding CoA-ester by one of the acyl-CoA synthetases and transported across the peroxisomal membrane in order to enter the peroxisomal β-oxidation for carbon chain shortening [163]. The medium- and short-chain acyl-CoAs that result from this oxidation (e.g. 4,8-dimethylnonanoyl-CoA, propionyl-CoA and acetyl-CoA) are converted to carnitine esters by CrOT and peroxisomal CrAT. This allows the export of these intermediates to the cytosol and further import into the mitochondria for complete oxidation. Alternatively, peroxisomal acyl-CoA esters are hydrolyzed by one of the peroxisomal acyl-CoA thioesterases [164], yielding the free acid and CoA. Only saturated unbranched and 2-methyl branched-chain fatty acids can directly undergo peroxisomal β-oxidation. This pathway follows a mechanism that involves dehydrogenation, hydratation, a second dehydrogenation step and thiolytic cleavage (Fig. 4), similarly to mitochondrial β-oxidation. The first dehydrogenation is catalyzed by one of the acyl-CoA oxidases (ACOX) present in humans, palmitoyl-CoA oxidase (active with straight-chain acyl-CoAs, mono and dicarboxylic-CoAs, and CoA esters derived from prostaglandins and xenobiotics) and branched-chain acyl-CoA oxidase (active with 2-methyl-branched acyl-CoAs and CoA esters of DHCA and THCA) [165]. The hydratation and second dehydrogenation steps are catalyzed by the L-bifunctional protein (LBP) or D-bifunctional protein (DBP), which display different substrate specificities [161, 162], and both contain enoyl-CoA hydratase and 3-hydroxyacyl-CoA dehydrogenase activities. The enzyme thiolase is responsible for the last step of the peroxisomal β-oxidation. Human peroxisomes contain two thiolases, a straight-chain 3-ketoacyl-CoA thiolase encoded by the *ACAA1* gene and the sterol carrier protein (SPC)-2/3-ketoacyl-CoA thiolase (SPCx), essential for the oxidation of pristanic acid (for review see [161]).

4. Branched-chain amino acid oxidation

The branched-chain amino acids (BCAA) leucine, isoleucine and valine are considered essential nutrients. Humans are not able to synthetize these amino acids and therefore they must be obtained from the diet [166]. Oxidation of BCAA (BCAAO) takes place in mitochondria, where these compounds are converted to organic acid intermediates that are able to enter the general metabolism. The first steps of BCAA metabolism are common to the three amino acids. BCAA can enter the mitochondria after transamination to their 2-keto acids in the cytosol or via a neutral amino acid carrier protein, being further transaminated in the mitochondrial matrix [167]. After oxidative decarboxylation of the branched-chain 2-keto acid by the branched-chain 2-keto acid dehydrogenase complex, branched-chain acyl-CoA thioesters are formed. From this step on, each BCAA follows its own pathway, that consists of distinct enzymes and which leads to the formation of different end products [167]. The degradation pathways of leucine, isoleucine and valine present many features in common with fatty acid β-oxidation (See Fig. 4 in Chapter 4).

4.1 Defects of branched-chain amino acid oxidation

Propionic and methylmalonic acidemia are the most frequent forms of branched-chain organic acidemias and are related to the valine and isoleucine degradation [168]. However, several other deficiencies are known from each of these pathways. Defects of leucine degradation include deficiency of the enzymes isovaleryl-CoA dehydrogenase (isovaleric acidemia), 3-methyl-crotonyl-CoA carboxylase, 3-methylglutaconyl-CoA

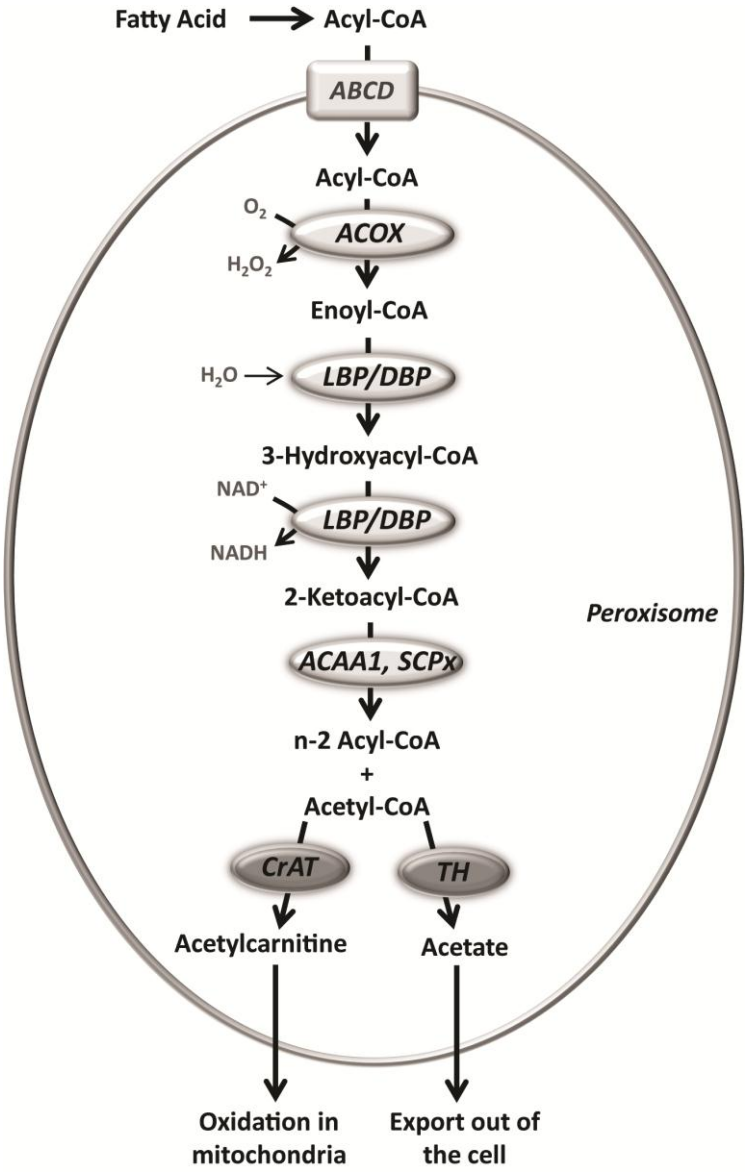

Figure 4 - Schematic representation of the peroxisomal fatty acid β-oxidation. After cytosolic esterification of fatty acids with coenzyme A (CoA), the respective acyl-CoAs probably enter the peroxisome for β-oxidation via an ABC-transporter (ABCD1, 2 or 3). The first step is catalyzed by one of the acyl-CoA oxidases (ACOX) present in humans, yielding an enoyl-CoA. Hydratation of this intermediate and the second dehydrogenation step are catalyzed by the L- or D-bifunctional protein (LBP or DBP) which contains the two activities. The resulting 3-ketoacyl-CoA is converted by the enzyme thiolase (ACAA1 or SPCx) into a 2-carbon chain-shortened acyl-CoA and acetyl-CoA. The acetyl-CoA released can be converted to acetylcarnitine by the peroxisomal carnitine acetyltransferase (CrAT) or undergo the thioesterase route (TH) with acetate as the major product.

hydratase (3-methylglutaconic acidemia) and 3-hydroxy-3-methylglutaryl-CoA lyase [169-172]. In the isoleucine pathway, deficiencies are found at the level of 2-methylbutyryl-CoA dehydrogenase, 2-methyl-3-hydroxybutyryl-CoA dehydrogenase and beta-ketothiolase [173-175]. Finally, the valine degradation pathway includes deficiencies at the level of isobutyryl-CoA dehydrogenase, 3-hydroxyisobutyryl-CoA hydrolase and methylmalonic semialdehyde dehydrogenase, as well as 3-hydroxyisobutyric aciduria [176-179].

5. Diagnosis of mFAO and BCAAO disorders and the importance of acylcarnitine profiling

Some of the clinical findings in mFAO and BCAAO deficient patients include the abnormal plasmatic free fatty acids and urinary organic acids. Due to the mFAO-blockage, the acyl-CoA and its β-oxidation intermediates accumulate intracellularly, being further metabolized by alternative oxidative pathways such as ω and ω-1 oxidation (leading to the production of dicarboxylic acids), and/or eliminated as acylglycines and acylcarnitines with secondary L-carnitine depletion. The dicarboxylic acids, acylglycines and acylcarnitines found in urine or plasma of patients are important biomarkers in the mFAO and BCAAO enzyme deficiencies. The relative amounts of the metabolites found in each deficiency depend on the affinities of the detoxifying enzymes for the various accumulated substrates [180]. Moreover, the fact that the characteristic abnormal metabolic profile is in most cases only detectable under metabolic decompensation often turns the biochemical diagnosis of many of these deficiencies into a difficult task. The introduction of the tandem mass spectrometry (MS/MS) as a tool for the diagnosis of inborn errors of metabolism was a crucial step in facilitating the diagnosis of mFAO and BCAAO disorders, among others. It allowed an accurate and precise determination of several metabolites, in particular acylcarnitines, allowing the establishment of characteristic profiles which are now used as biomarkers in neonatal screening programs. Nonetheless, the etiology of these acylcarnitines is still not completely elucidated. It is generally assumed that the observed acylcarnitine profiles reflect the intramitochondrially accumulating acyl-CoAs exported out of the mitochondria as acylcarnitines [181]. This would involve the synthesis of acylcarnitines within the mitochondrial matrix followed by the mitochondrial and cellular export of these derivatives, processes not yet truly clarified. The diagnosis of these diseases must always be complemented with the determination of the enzymatic or transport activity of the suspected deficient protein in lymphocytes, leukocytes or fibroblasts of the patient and the molecular characterization of the disease-causing mutation in the corresponding gene(s).

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Chapter 3

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

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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 *Δcat2* (*cat2*::KAN) and *Δfox2* (*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 *Δcat2* and *Δfox2* 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 *Δcat2 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-

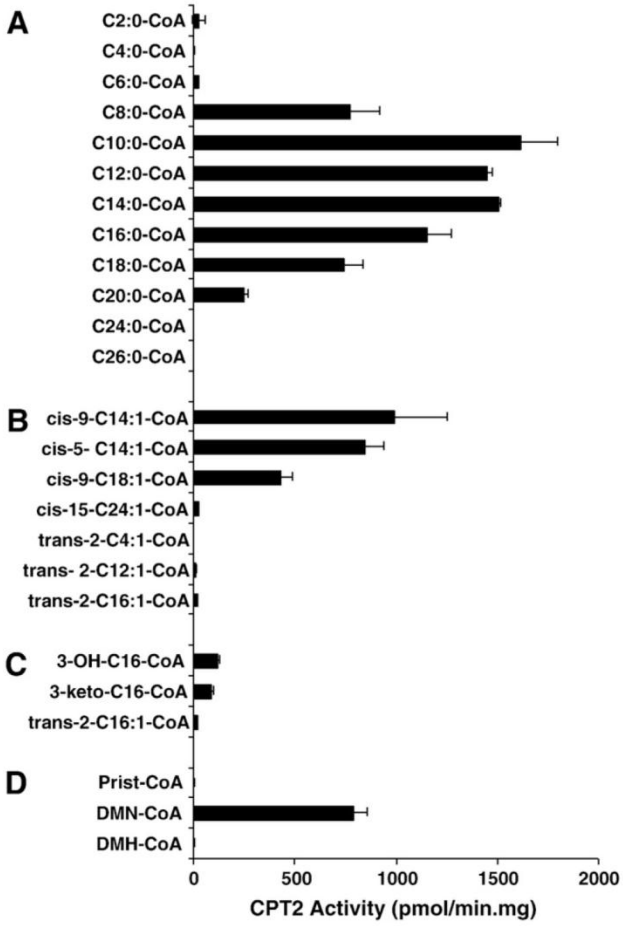

Fig. 1. CPT2 activity with straight-chain acyl-CoA esters (A), unsaturated intermediates (B), C16 mFAO intermediates (C) and peroxisomal products and intermediates (D). The reactions were measured at 37 °C for 10 min in a standard incubation medium described in Materials and methods with L-carnitine and 25 μM of each acyl-CoA ester. The produced acylcarnitines were quantified after derivatization with 1-propanol/acetylchloride by ESI-MS/MS. For additional experimental details see Materials and methods. Data shown are mean ± S.D. of duplicates of at least two independent experiments.

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 amino acids 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 (Prist-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).

Table 1
Evaluation of CPT2 activity with acyl-CoAs formed upon branched-chain amino acids 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

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 ($K_{m(C16\text{-}CoA)} = 7.1$ μM and $K_{m(trans\text{-}2\text{-}C16:1\text{-}CoA)} = 8.1$ μM), 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 catalyzed by CPT2, inhibition studies were performed in the Δ*fox2 S. cerevisiae* mutant (BY4742 *fox2*::KAN) transformed with the plasmid described in Section2.2 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 → 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

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/acethylchloride by ESI-MS/MS. Values were calculated from Lineweaver–Burk plots (not shown).

	C16:0-CoA	*trans*-2-C16:1-CoA
K_m^{app} (μM)	7.1	8.1
V_{max}^{app} (pmol/min.mg)	1156	77
V_{max}/K_m	163	9.5

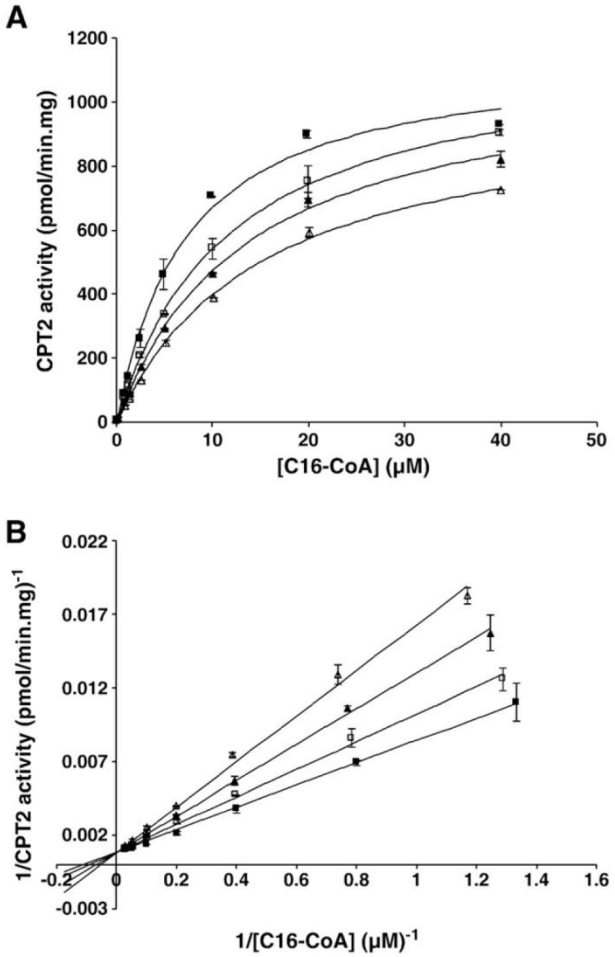

Fig. 2. Evaluation of CPT2 activity using C16-CoA as substrate (0 to 40 μM) in the presence of *trans*-2-C16:1-CoA. Inhibition studies were performed with 0 to 40 μM of *trans*-2-C16:1-CoA in the presence of 0,1% BSA. A) Non-linear regression; B) Lineweaver–Burk (0 μM; 5 μM; 10 μM; 20 μM *trans*-2-C16:1-CoA). The reactions were measured at 37 °C for 5 min in a standard incubation medium described in Materials and methods. The produced acylcarnitines were quantified by UPLC-MS. For additional experimental details see Materials and methods. Non-linear regression analysis was performed using the Berkley Madonna™ software.

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 (Fig. 2; Table 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-dimethyl-heptanoyl-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 amino acid 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

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.

	Trans-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

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 amino acid 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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Chapter 4

Substrate specificity of human carnitine acetyltransferase: implications for fatty acid and branched-chain amino acid metabolism

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Substrate specificity of human carnitine acetyltransferase: implications for fatty acid and branched-chain amino acid metabolism

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Abstract

Carnitine acyltransferases catalyze the reversible conversion of acyl-CoAs into acylcarnitine esters. This family includes the mitochondrial enzymes carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltransferase (CrAT). CPT2 is part of the carnitine shuttle that is necessary to import fatty acids into mitochondria and catalyzes the conversion of acylcarnitines into acyl-CoAs. In addition, when mitochondrial fatty acid β-oxidation is impaired, CPT2 is able to catalyze the reverse reaction and converts accumulating long- and medium-chain acyl-CoAs into acylcarnitines for export from the matrix to the cytosol. However, CPT2 is inactive with short-chain acyl-CoAs and intermediates of the branched-chain amino acid oxidation pathway (BCAAO). In order to explore the origin of short-chain and branched-chain acylcarnitines that may accumulate in various organic acidemias, we performed substrate specificity studies using purified recombinant human CrAT. Various saturated, unsaturated and branched-chain acyl-CoA esters were tested and the synthesized acylcarnitines were quantified by ESI-MS/MS. We show that CrAT converts short- and medium-chain acyl-CoAs (C2 to C10-CoA), whereas no activity was observed with long-chain species. *Trans*-2-enoyl-CoA intermediates were found to be poor substrates for this enzyme. Furthermore, CrAT turned out to be active towards some but not all the BCAAO intermediates tested and no activity was found with dicarboxylic-CoA esters. This suggests the existence of another enzyme able to handle the acyl-CoAs that are not substrates for CrAT and CPT2, but for which the corresponding acylcarnitines are well recognized as diagnostic markers in inborn errors of metabolism.

Keywords: Acylcarnitines; Fatty acid oxidation; Branched-chain amino acid oxidation; Enoyl-CoA intermediates; Carnitine shuttle.

Introduction

Carnitine acyltransferases catalyze the transfer of acyl groups between coenzyme A (CoA) and carnitine. The carnitine acyltransferase family is crucial in the catabolism of fatty acids and includes four members: carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2), carnitine octanoyltransferase (CrOT) and carnitine acetyltransferase (CrAT). Prior to mitochondrial β-oxidation, long-chain fatty acids are activated to CoA esters. However, these large acyl-CoAs are unable to cross the inner mitochondrial membrane and have to be converted into the respective carnitine ester by CPT1, localized within the outer mitochondrial membrane. Carnitine/acylcarnitine translocase (CACT) is responsible for the transport of the long-chain acylcarnitines across the inner mitochondrial membrane whereas CPT2, associated with the inner

Abbreviations: BCAAO, branched-chain amino acid oxidation; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; CrAT, carnitine acetyltransferase; CrOT, carnitine octanoyltransferase; mCrAT, mitochondrial CrAT; mFAO, mitochondrial fatty acid beta-oxidation; pCrAT, peroxisomal CrAT.

aspect of the inner mitochondrial membrane, catalyzes their reconversion to acyl-CoAs, which can then undergo β-oxidation [1].

The other two members of the acyltransferase family, CrAT and CrOT have preference for short- and medium-chain substrates, respectively. Both are soluble proteins found in peroxisomes but while CrOT only exists in this organelle, CrAT is also found in the mitochondrial matrix [2-5]. Very long-chain, branched-chain and long-chain dicarboxylic fatty acids undergo β-oxidation in the peroxisomes. The medium- and short-chain acyl-CoAs that result from this oxidation are converted to carnitine esters, by CrOT and peroxisomal CrAT. This allows the export of these intermediates to the cytosol and further import into the mitochondria for complete oxidation [6]. In mitochondria CrAT has a role in the maintenance of the acyl-CoA/CoA pool [6, 7] and it has been recently described as a modulator of whole body glucose homeostasis by affecting pyruvate dehydrogenase flux in a muscle-specific CrAT knockout mouse model [8]. The structural studies performed with the human CrAT and the resolution of the 3D structure of this enzyme were major advances in the understanding of the catalytic mechanism of acyltransferases enzyme class [9, 10].

It is now established that CPT2 is able to produce a net flux in the reverse direction, converting medium- and long-chain acyl-CoAs into the respective acylcarnitine intermediates [11-13]. This is particularly important in patients with a genetic or acquired mitochondrial fatty acid β-oxidation (mFAO) disorder, where acyl-CoAs accumulate intramitochondrially. CPT2 catalyzes the synthesis of these compounds into acylcarnitines that are further exported to the cytosol by CACT [14]. These acylcarnitines are ultimately exported to the plasma via a still unknown mechanism and are important biomarkers of inborn errors affecting mFAO or branched-chain amino acid oxidation (BCAAO) [15, 16].

While CPT2 is responsible for the synthesis of medium- and long-chain acylcarnitines [11-13], the short- and branched-chain acylcarnitine intermediates require another acyltransferase for their synthesis. CrAT from mouse liver and pigeon breast as well as the recombinant CrAT from rat, have been described to catalyze the synthesis of carnitine esters from acyl-CoAs with chain length from C2 to C10-CoA [17-20] and the human enzyme (purified from liver) also shows some activity with long-chain substrates (C12 to C14-CoA) [21]. The lack of a thorough study on the human forms of CrAT prompted us to further investigate the substrate specificity of this enzyme, not only for the straight-chain acyl-CoAs, but also for the BCAAO intermediates. In this paper we present experimental evidence that provide new insights into the role of CrAT in the formation of acylcarnitines. Furthermore we could confirm that *trans*-2-enoyl-CoAs are poor substrates for the enzymes from the carnitine acyltransferase family.

Materials and Methods

Materials

The Zymoclean gel DNA recovery kit was purchased from Zymo Research (Irvine, California, USA). The *Escherichia coli* strain BL21(DE3) was obtained from Invitrogen (Carlsbad, California, USA). Peptone and yeast extract were obtained from Difco Laboratories Inc. (Detroit, MI). The HisLink™ protein purification resin was obtained from Promega (Madison, USA). The substrate *trans*-2-C5:1-CoA was enzymatically synthesized as described by Rasmussen *et al* [22]. All other CoA esters were obtained from Sigma-Aldrich (St. Louis, MO, USA) and prepared in MES buffer, pH 6.0. Human serum albumin, bovine serum albumin, L-carnitine and acetylchloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Construction of the expression plasmids

The sequences corresponding to the mitochondrial (amino acids 30 to 626) and peroxisomal (amino acids 22 to 626) CrAT (mCrAT and pCrAT, respectively) were amplified by PCR using human fibroblast cDNA as a template. The forward primers were designed including an *Nde*I restriction site (5'-TATATCATATGAAGGCTTCCAGCCGCTTCAA-3' and 5'-TATATCATATGGCACACCAGGATGCACTGCCACG-3', respectively for the mCrAT and pCrAT). The same reverse primer was used for both mCrAT and pCrAT (5'- TATATGGATCCTCCTAGGGGCTCAGAGCTTG -3'). The PCR product was first cloned in the pGEMT vector. After digestion with *Nde*I and *Sal*I (located in pGEM-T) the product was cloned in frame with an N-terminal histidine tag between the *Nde*I and *Xho*I sites of the pET19b expression vector. Confirmation

of the sequence was performed by direct sequencing and the plasmids were used to transform *E.coli* BL21(DE3).

Heterologous expression and purification of the mitochondrial and peroxisomal carnitine acetyltransferases

Histidine tagged mCrAT and pCrAT were expressed in *E. coli* BL21(DE3) in Terrific Broth medium (12 g/L tryptone, 24 g/L yeast extract, 8 g/L glycerol and 25 mM potassium phosphate buffer pH 7.5) for 16 hours at 22ºC. The enzymes were purified on HisLink protein purification resin according to the manufacurer's protocol. The presence of the protein in each fraction was accessed by immediately measuring the activity which was quantified as the reduction of 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB). The reaction mixture contained 100 mM Tris-HCl pH 8.0, 1 mM EDTA, 5 mM L-carnitine, 0.2 mM DTNB and 20 µL of purified protein 50 times diluted. The reaction was started by the addition of acetyl-CoA at a final concentration of 0.2 mM. The reduction of DTNB was followed in time on a spectrophotometer (Uvicon) at 412 nm. The fractions containing CrAT activity were pooled and protein concentration was determined using the bicinchoninic acid assay [23] and human serum albumin as standard. Glycerol 50% was added to the enzyme fractions which were stored at -80ºC.

Determination of mitochondrial and peroxisomal carnitine acetyltransferase substrate specificities

Carnitine acetyltransferase activity was determined using the method described by van Vlies and co-workers for CPT1 [24]. The standard assay 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 to a final volume of 150 µL. The reaction was initiated by the addition of 5 µL of enzyme (approximately 30 ng of protein per assay) and was allowed to proceed at 37ºC. After 10 min incubation, the reaction was terminated by adding 750 µL acetonitrile containing 50 pmol d3-propionylcarnitine (C3), 50 pmol d3-octanoylcarnitine (C8) and 25 pmol d3-palmitoylcarnitine (C16) internal standards. After derivatization of the produced acylcarnitines with 1-butanol/acetylchloride 4/1 (v/v), these intermediates were quantified by Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS). The purity of the acyl-CoAs was initially confirmed by reverse phase high performance liquid chromatography (HPLC). The data obtained by ESI-MS/MS for all the short branched-chain acyl-CoAs was confirmed by ultra-performance liquid chromatography tandem MS (UPLC-MS/MS), essentially as described elsewhere [25].

Determination of the kinetic parameters of mitochondrial and peroxisomal carnitine acetyltransferases

The kinetic parameters (*K*m and *V*max) for mitochondrial and peroxisomal CrAT were determined using C2-CoA, C4-CoA and *trans*-2-C4:1-CoA as substrates by measuring their activities in the absence and presence of different concentrations of each substrate (0 – 1.3 mM). Activity was assessed as described above. Non-linear regression was determined with the GraphPad Prism 5 software and the Michaelis-Menten equation was used to determine the kinetic parameters.

Results

Determination of the substrate specificity of the mitochondrial and peroxisomal carnitine acetyltransferases

We used recombinant human mitochondrial and peroxisomal CrAT (mCrAT and pCrAT, respectively) for the determination of the substrate specificity of both isoforms. Both mCrAT and pCrAT revealed identical substrate specificity profiles for all substrates and therefore we only show the results obtained with mCrAT. CrAT was found to be active towards short- (C2-C6) and medium-chain (C8-C10) acyl-CoAs (Fig. 1). The enzyme did show some activity with acyl-CoAs of longer chain length including C12- and C14-CoA, but not with C16-CoA (Fig. 1). Interestingly, *trans*-2-enoyl-CoAs, including mFAO and BCAAO intermediates such as *trans*-2-ene-C_4-CoA (crotonyl-CoA, *trans*-2-C4:1-CoA), *trans*-2-ene-3-methyl-C_4-CoA (3-methylcrotonyl-CoA), and *trans*-2-ene-2-methyl-C_4-CoA (tiglyl-CoA, *trans*-2-C5:1-CoA), were found to be poor substrates for

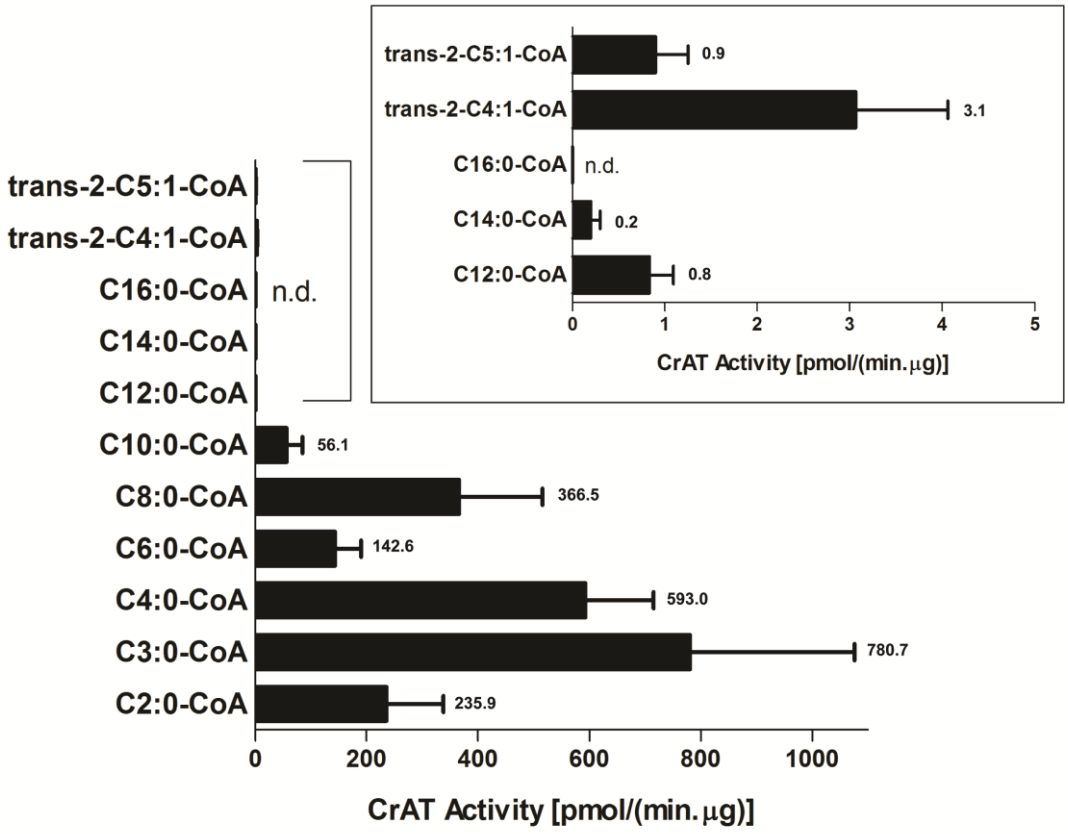

Fig. 1. Mitochondrial CrAT (mCrAT) activity with saturated and unsaturated straight-chain acyl-CoAs. The value in front of each bar corresponds to the mean activity expressed in pmol/(min.μg) for the acyl-CoA tested. The symbol n.d. denotes below the limit of detection. Error bars indicate the standard deviation (SD). Data shown are mean ± SD of duplicates of three independent experiments.

CrAT when compared with the straight-chain C4-CoA intermediate (Fig. 1, and 2).

The degradation pathways of the different BCAA involve various branched-chain acyl-CoA intermediates. We tested the activity of CrAT with several of these compounds as well as with some intermediates involved in dicarboxylic acid oxidation, peroxisomal β-oxidation and ketone bodies synthesis (Fig. 2). CrAT was found to be active with most of the substrates tested. The branched-chain acyl-CoAs had a much lower affinity towards CrAT than the straight-chain equivalents (Fig. 1 and 2). Remarkably, CrAT did not display activity with 3-hydroxy-3-methylglutaryl-CoA, 2-methylacetoacetyl-CoA and dicarboxylic acyl-CoAs (malonyl-, methylmalonyl-, succinyl- and glutaryl-CoA) (Fig. 2).

Determination of the kinetic parameters of mitochondrial and peroxisomal CrAT

We determined the kinetic parameters of mCrAT and pCrAT (*K*m and *V*max) using acetyl-CoA (C2-CoA) as a substrate. Facing the fact that enoyl-CoAs are poor substrates for CrAT, we performed a comparative analysis of the kinetic parameters of the enzyme with C4:0-CoA versus *trans*-2-C4:1-CoA. Employing non-linear regression to the Michaelis-Menten equation, we found that mCrAT has lower affinity towards C2-CoA (*K*m = 240 μM) than the peroxisomal enzyme pCrAT (*K*m = 78 μM) (Table 1). The affinity of both enzymes for C4:0-CoA and *trans*-2-C4:1-CoA was in the same order of magnitude. However, the catalytic efficiency (*V*max/*K*m) of the enzyme with *trans*-2-C4:1-CoA was approximately 100-fold lower than for C4:0-CoA (Table 1). Similar results were observed with the peroxisomal enzyme (results not shown).

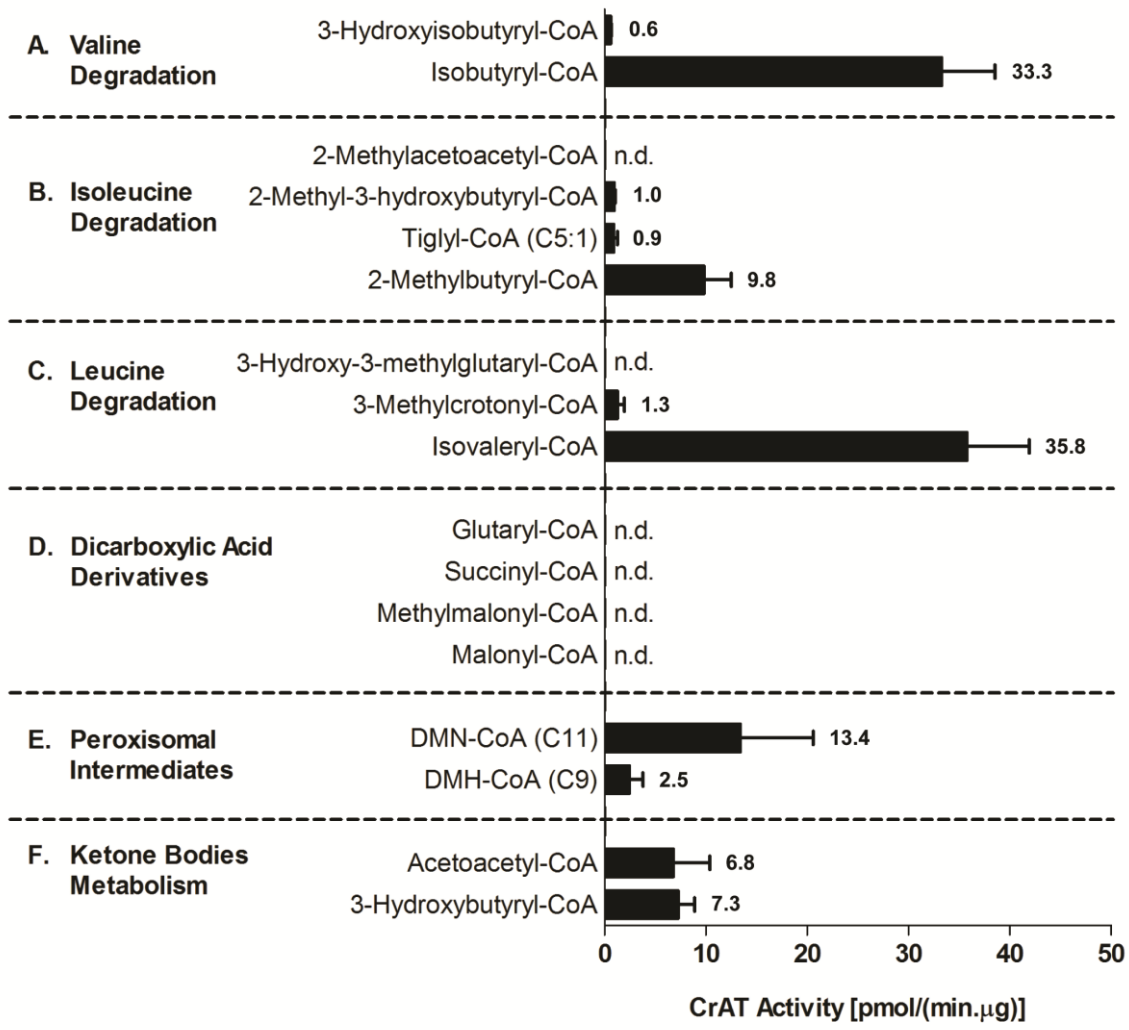

Fig. 2. Mitochondrial CrAT (mCrAT) activity with acyl-CoAs from A) valine degradation pathway; B) isoleucine degradation pathway; C) leucine degradation pathway; D) dicarboxylic acids derivatives; E) peroxisomal intermediates and F) ketone bodies' metabolism. The value in front of each bar corresponds to the mean activity expressed in pmol/(min.μg) for the acyl-CoA tested. The symbol n.d. denotes below the limit of detection. Error bars indicate the standard deviation. Data shown are mean ± SD of duplicates of three independent experiments.

Discussion

In the light of its crucial role in mFAO, the carnitine acyltransferase family has been extensively studied throughout the years. A particularly large number of reports have been published concerning the function and structure of carnitine acetyltransferase [6, 8-10, 26, 27]. However, most of what is known about its substrate specificity was investigated with non-human sources of the enzyme [17-20] or was exclusively focused on the straight-chain substrates [21]. In patients suffering from mFAO and BCAAO disorders, the reverse acyltransferase reaction is a critical step for the clearance of the accumulating acyl units within the mitochondria. We recently demonstrated in an *in vitro* system that CPT2 is able to reverse the direction of the enzyme reaction for medium- and long-chain acyl-CoAs but no activity is found with short-chain acyl-CoAs or with the BCAAO intermediates [13]. However, some of these straight short-chain and branched-chain acylcarnitines are commonly found in the plasma of patients with mFAO and BCAAO disorders and may be used as biomarkers for the newborn screening and further diagnosis. The proposed candidate to synthetize these short- and branched-chain acylcarnitines is CrAT, the other acyltransferase known to be present in mitochondria in addition to CPT2. Therefore, in order to clarify the origin of these metabolites, we performed substrate

Table 1. Kinetic constants of mitochondrial and peroxisomal CrAT (mCrAT and pCrAT, respectively), using acetyl-CoA (C2-CoA) as a substrate. Comparative analysis of the kinetic constants and catalytic efficiency (*V*max/*K*m) of mCrAT with butyryl-CoA (C4:0-CoA) versus *trans*-2-butenoyl-CoA (*trans*-2-C4:1-CoA). Analysis was performed with the GraphPad Prism 5 software using the non-linear regression of the Michaelis-Menten equation. Data shown are mean ± SD of duplicates of at least two independent experiments.

Protein	Substrate	*K*m (μM)	*V*max [nmol/(min.μg)]	*V*max/*K*m [1/(min.μg)]
mCrAT	C2-CoA	240 ± 47	2.5 ± 0.2	-
pCrAT	C2-CoA	78 ± 18	4.4 ± 0.3	-
mCrAT	C4-CoA	499 ± 44	10.7 ± 0.4	$2.1x10^{-5}$
mCrAT	*trans*-2-C4:1-CoA	590 ± 129	0.13 ± 0.01	$2.2x10^{-7}$

specificity studies using purified recombinant human mitochondrial and peroxisomal CrAT. As described previously [21], we found that the human mCrAT handles acyl-CoAs with a chain length ranging from 2 - 14 carbon atoms, with C3-CoA as the most efficient substrate (Fig. 1). Surprisingly, the activity of mCrAT with C6-CoA was consistently lower than with C8-CoA. The opposite has been found for glycine-*N*-acyltransferase which has virtually no activity with C8 [28]. We could not find an explanation for this finding which nevertheless was already reported for the mouse enzyme [18, 19]. Mitochondrial CRAT had hardly any activity with C12- and C14-CoA, and had no detectable activity with C16-CoA (Fig. 1). The pCrAT behaved essentially as the mitochondrial enzyme, showing an identical substrate specificity profile (results not shown). Interestingly, acyl-CoAs with the same chain-length but with a *trans* double bond at the C2 position turned out to be poor substrates for CrAT, showing a catalytic efficiency approximately 100-fold lower than the corresponding straight-chain acyl-CoA (Table 1). We had previously observed a similar phenomenon for CPT2, suggesting that the *trans*-2-enoyl-CoA intermediates may interfere with the catalytic mechanism of acyltransferases [13]. The catalytic mechanism of acyltransferases involves a nucleophilic attack that leads to the formation of a tetrahedral intermediate anion (Fig. 3A) (for review see [29]). The existence of a double bond at the second position may cause resonance and lead to a distribution of the positive charge, which makes the carbon atom of the carbonyl group less positive. The charge displacement will likely hamper the nucleophilic attack that always takes place at a positive center (Fig. 3B). This could lead to the inhibition of the tetrahedral intermediate formation which consequently inhibits the acylcarnitines synthesis. We speculate that this interference may explain why the *trans*-2-enoyl-CoAs are poor substrates for CPT2 and CrAT. A similar decrease in CrAT activity was also observed with acyl-CoAs in which a methyl group is located at the second position. This is evidenced when comparing the activity obtained with the substrates 2-methylacetoacetyl-CoA and 2-methylbutyryl-CoA with acyl-CoAs of similar structure but lacking the methyl group at the C2 position (acetoacetyl-CoA and isovaleryl-CoA, respectively) (Fig. 2). Therefore, similarly to *trans*-2-enoyl-CoAs, we suggest that a methyl group located at the second carbon of the acyl-unit may interfere with the catalytic mechanism of CrAT.

The carnitine acetyltransferases displayed activity with some but not all the branched-chain acyl-CoAs tested. Isobutyryl-CoA, a metabolite in the degradation of valine (Fig. 4), accumulates in patients with isobutyryl-CoA dehydrogenase deficiency as well as in patients with the multiple acyl-CoA dehydrogenation defect (MADD). The isobutyryl- moiety is found in the plasma of these patients as the respective carnitine intermediate, i.e. isobutyrylcarnitine [30], which according to our results can be synthesized by CrAT (Fig. 2A). CrAT is also able to convert 3-hydroxyisobutyryl-CoA into the respective 3-hydroxyisobutyrylcarnitne (Fig. 2A). This intermediate is characteristic of 3-hydroxyisobutyryl-CoA hydrolase deficiency [31].

Among the acyl-CoAs of the isoleucine degradation pathway (Fig. 4), the highest CrAT activity was observed with 2-methylbutyryl-CoA (Fig. 2B). This is the intermediate that accumulates in short branched-chain acyl-CoA dehydrogenase (SBCAD) deficiency and the respective acylcarnitine is the biomarker for this disease [32]. Although tiglyl-CoA and 2-methyl-3-hydroxybutyryl-CoA were found to be poor substrates for CrAT, we did find some conversion to the respective acylcarnitines (Fig. 2B). These acylcarnitine intermediates are usually found in the plasma of patients suffering from 2-methyl-3-hydroxybutyryl-CoA dehydrogenase deficiency [33, 34] and according to our results, CrAT may be the enzyme responsible for their formation. The metabolite 2-methylacetoacetylcarnitine has never been reported in the plasma of patients with β-ketothiolase deficiency, which is in line with our finding that CrAT is not active with this compound.

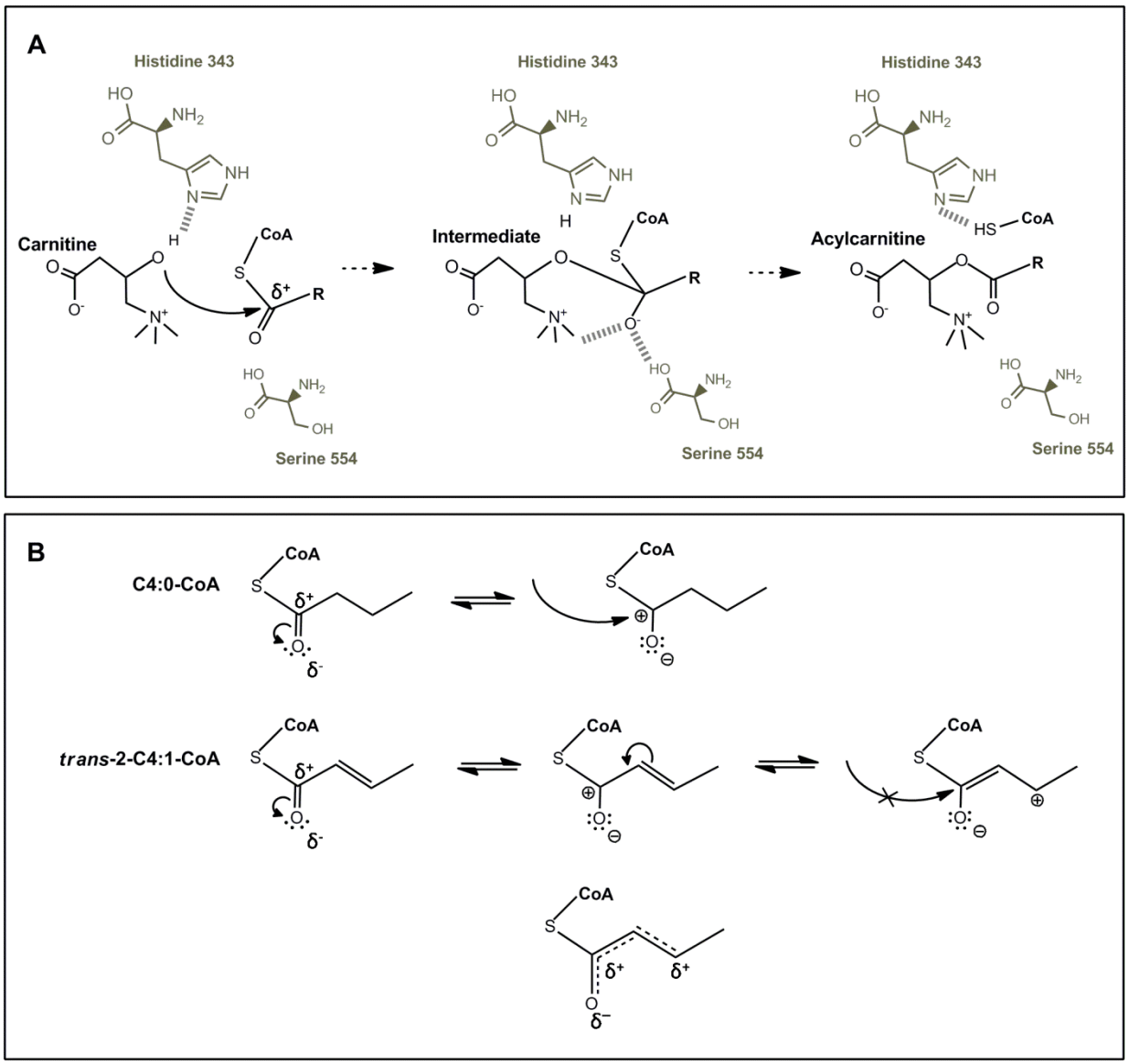

Fig. 3. Schematic representation of the catalytic mechanism of acyltransferases. A) Mechanism of acylcarnitines formation. The catalytic histidine 343 acts as a general base to extract the proton from the hydroxyl group of carnitine or the thiol group of CoA supporting the nucleophilic attack between carnitine and the acyl-CoAs. The tetrahedral intermediate formed is probably stabilized by the interaction of the oxyanion with both carnitine and the hydroxyl group of serine 554. Adapted from Jogl *et al* [29]. B) Detail of the nucleophilic group of the substrates C4:0-CoA and *trans*-2-C4:1-CoA. The double bond in the enoyl-CoA intermediate may cause resonance in the molecule which leads to the displacement of the positive charge and hampers the nucleophilic attack (crossed arrow).

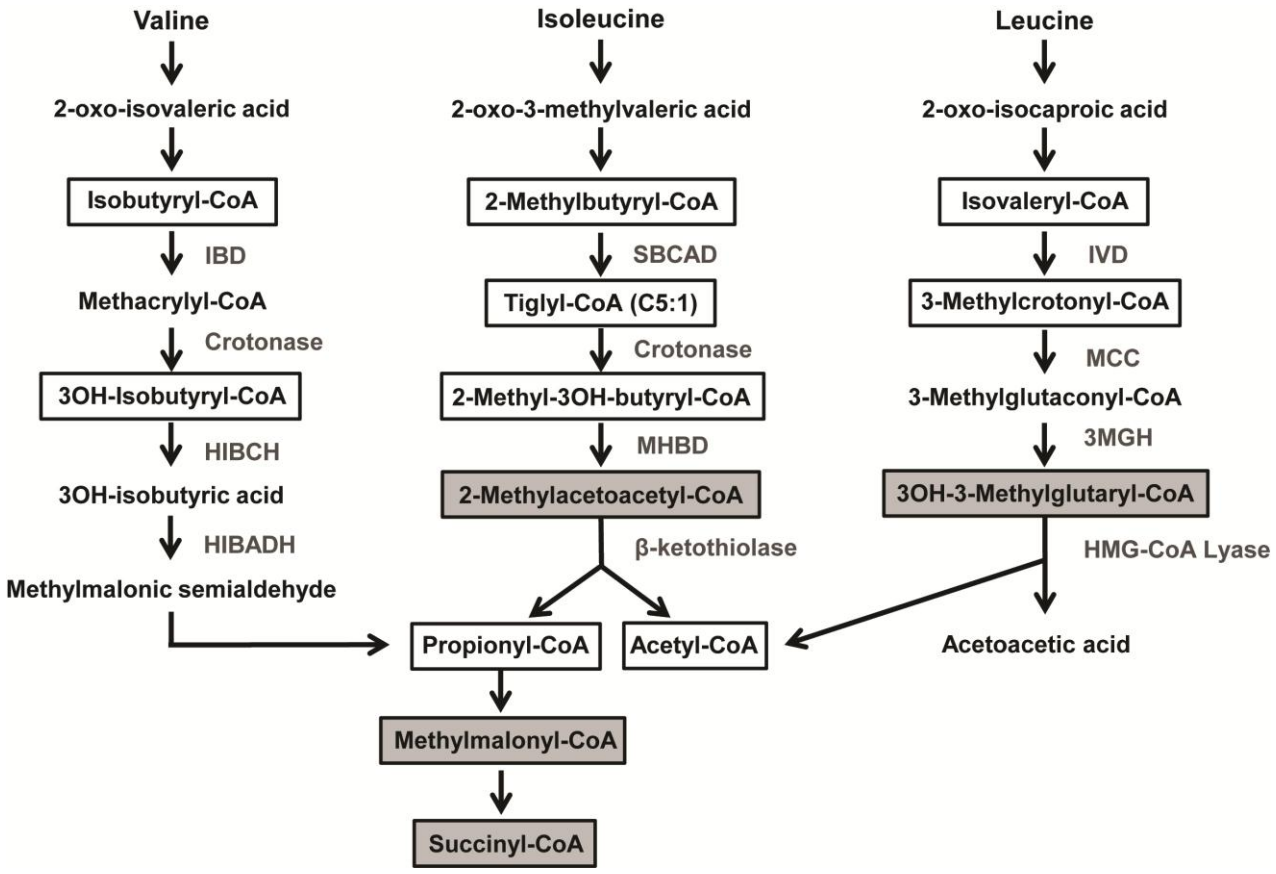

Fig. 4. Catabolic pathways of the branched-chain amino acids valine, isoleucine and leucine. The substrates for which CrAT activity was tested are in white boxes and the ones that are not substrates for the enzyme are represented in grey boxes. The names of the enzymes are shown on the right. IBD: isobutyryl-CoA dehydrogenase; HIBCH: 3-hydroxyisobutyryl-CoA dehydrogenase; HIBADH: 3-hydroxyisobutyrate dehydrogenase; SBCAD: short branched-chain acyl-CoA dehydrogenase; MHBD: 2-methyl-3-hydroxybutyryl-CoA dehydrogenase; IVD: isovaleryl-CoA dehydrogenase; MCC: 3-methylcronotyl-CoA carboxylase; 3MGH: 3-methylglutaconyl-CoA hydratase; HMG-CoA Lyase: 3-hydroxy-3-methylglutary-CoA lyase

Isovaleryl-CoA is a metabolite in the leucine degradation pathway (Fig. 4) and accumulates in cases of isovaleryl-CoA dehydrogenase deficiency (isovaleric acidemia), which can be diagnosed by the elevation of isovalerylcarnitine in plasma [35, 36]. Indeed, isovaleryl-CoA was shown to be a good substrate for CrAT (Fig. 2C), which indicates that this is the enzyme likely responsible for its conversion to the corresponding acylcarnitine. Concerning the remaining intermediates of the leucine pathway, CrAT showed some activity towards 3-methylcrotonyl-CoA but none when 3-hydroxy-3-methylglutaryl-CoA (HMG-CoA) was studied (Fig. 2C). Unfortunately, 3-hydroxy-isovaleryl-CoA and 3-methylglutaconyl-CoA were not available and thus could not be tested. All neonatal screening programs use 3-hydroxy-isovalerylcarnitine as a marker of 3-methylcrotonyl-CoA carboxylase deficiency but the mechanism by which it is formed has not been elucidated so far. Patients with a deficiency of HMG-CoA lyase do not accumulate HMG-carnitine but are characterized by measurable quantities of 3-methylglutarylcarnitine and 3-hydroxyisovalerylcarnitine. To our knowledge no report on the occurrence of 3-methylglutaconyl-carnitine has ever been reported.

We also investigated CrAT activity with short-chain dicarboxylic acyl-CoAs (Fig. 2D). Unexpectedly, no activity was found with any of the substrates tested (malonyl-, methylmalonyl-, succinyl- and glutaryl-CoA). The respective acylcarnitines are commonly found in several disorders such as malonic acidemia (malonyl-CoA decarboxylase deficiency), methylmalonic acidemia (methylmalonyl-CoA mutase deficiency), combined malonic and methylmalonic acidemia (with mutations in the

gene *ACSF3*), succinyl-CoA ligase deficiency (due to mutations in the *SUCLG1* and *SUCLA2* genes) and glutaric acidemia (glutaryl-CoA dehydrogenase deficiency) [37-41]. Therefore, as CrAT does not seem to be responsible for the formation of these acylcarnitines, another enzyme must handle the intermediates accumulating in the above mentioned inherited metabolic disorders. In order to investigate if dicarboxylic acylcarnitines could be produced using another enzyme source, we incubated human liver and kidney homogenates with each of the dicarboxylic acyl-CoAs. Surprisingly, none of the expected dicarboxylic acylcarnitines were detected (results not shown). Obviously further investigations are necessary to resolve this issue.

Finally, we tested some peroxisomal intermediates derived from pristanic acid oxidation and some ketone bodies derivatives. Peroxisomal β-oxidation of pristanic acid yields the intermediate 4,8-dimethylnonanoyl-CoA (DMN-CoA), probably converted by CrOT into its carnitine ester [42] and exported to the cytoplasm. Further oxidation requires the mitochondrial import of DMN-carnitine and its reconversion to DMN-CoA. One additional cycle of β-oxidation in mitochondria leads to the formation of 2,6-dimethylheptanoyl-CoA (DMH-CoA). We previously demonstrated that CPT2 is active with DMN-CoA but not with DMH-CoA [13]. Now, we show that both substrates are converted to the respective acylcarnitine by CrAT (Fig. 2E). The ketone bodies derivatives acetoacetyl-CoA and 3-hydroxybutyryl-CoA were also tested and CrAT was found to be active with both substrates (Fig. 2F). The carnitine ester 3-hydroxybutyrylcarnitine was proposed to be an important player in insulin resistance in mice [41] and has recently been described as a metabolite in ketone bodies' metabolism [42]. Furthermore, L-3-hydroxy-butyrylcarnitine is also known to accumulate in short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) deficient patients [45]. We observed that mCrAT mainly produced L-3-hydroxy-butyrylcarnitine from a racemic mixture of D- and L-3-hydroxybutyryl-CoA, suggesting that its activity is higher with the L-isoform.

Conclusions

In summary, both mitochondrial and peroxisomal CrAT have been found to be able to catalyze the acylcarnitine synthesis from straight-chain acyl-CoAs with chain length up to ten carbon atoms. Furthermore, we have shown that *trans*-2-enoyl-CoA intermediates are poor substrates for CrAT, similarly to what was previously reported for CPT2, thus suggesting that the catalytic mechanism of acyltransferases is hampered by the presence of a double bond at the second position of the substrate carbon chain. We further show that CrAT is active towards the intermediates of the BCAAO pathway that are commonly found in the acylcarnitine profiles of patients and with some peroxisomal intermediates derived from pristanic acid oxidation. For the dicarboxylic acyl-CoA species that do not seem to be handled by CrAT, but for which acylcarnitines are found in the plasma of patients affected with BCAAO deficiencies, further studies are necessary to disclose their underlying mode of synthesis.

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Chapter 5

Carnitine palmitoyltransferase 2 and carnitine/acylcarnitine translocase are involved in the mitochondrial synthesis and export of acylcarnitines

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Carnitine palmitoyltransferase 2 and carnitine/acylcarnitine translocase are involved in the mitochondrial synthesis and export of acylcarnitines

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ABSTRACT **Acylcarnitines are commonly used in the diagnosis of mitochondrial fatty acid β-oxidation disorders (mFAODs). It is generally assumed that this plasma acylcarnitine profile reflects the mitochondrial accumulation of acyl-CoAs. The identity of the enzymes and the mitochondrial and plasmalemmal transporters involved in the synthesis and export of these metabolites have remained undefined. We used lentiviral shRNA to knock down the expression of medium-chain acyl-CoA dehydrogenase (MCAD) in control and carnitine palmitoyltransferase 2 (CPT2)-, carnitine/acylcarnitine translocase (CACT)-, and plasmalemmal carnitine transporter (OCTN2)-deficient human fibroblasts. These cell lines, including mock-transduced controls, were loaded with decanoic acid and carnitine, followed by the measurement of the acylcarnitine profile in the extracellular medium. In control fibroblasts, MCAD knockdown markedly increased the production of octanoylcarnitine (3-fold, $P<0.01$). OCTN2-deficient cell lines also showed extracellular accumulation of octanoylcarnitine (2.8-fold, $P<0.01$), suggesting that the cellular export of acylcarnitines does not depend on OCTN2. In contrast, in CPT2- and CACT-deficient cells, the accumulation of octanoylcarnitine in the medium did not significantly increase in the MCAD knockdown. Similar results were obtained using pharmacological inhibition of CPT2 in fibroblasts from MCAD-deficient individuals. This shows that CPT2 and CACT are crucial for mitochondrial acylcarnitine formation and export to the extracellular fluids in mFAOD.—Violante, S., IJlst, L., te Brinke, H., Tavares de Almeida, I., Wanders, R. J. A., Ventura, F. V., Houten, S. M. Carnitine palmitoyltransferase 2 and carnitine/acylcarnitine translocase are involved in the mitochondrial synthesis and export of acylcarnitines. *FASEB J.* 27, 000–000 (2013). www.fasebj.org**

Key Words: mitochondrial fatty acid β-oxidation · Acyl-CoA metabolites

Mitochondrial fatty acid β-oxidation (mFAO) is an important source of energy production in mammals, especially during prolonged periods without feeding. It is particularly important in the heart and skeletal muscle, tissues that largely depend on the oxidation of long-chain fatty acids to maintain their energy requirements (1). The breakdown of fatty acids takes place mainly in the mitochondria, where the generation of acetyl-coenzyme A (CoA) and reducing equivalents (NADH and $FADH_2$) provides the necessary substrates for ATP production.

In contrast to medium-chain fatty acids that are assumed to cross the mitochondrial membranes without prior activation to acyl-CoA esters, long-chain fatty acids have to be converted into acyl-CoAs followed by entry into the mitochondrion *via* the so-called carnitine shuttle. The carnitine shuttle is composed by the enzymes carnitine palmitoyltransferase 1 and 2 (CPT1 and CPT2) and the carrier carnitine/acylcarnitine translocase (CACT). Activated acyl units are converted to acylcarnitines by CPT1 and further transported across the inner mitochondrial membrane (IMM) by CACT. The enzyme CPT2, bound to the inner aspect of the IMM, is responsible for the conversion of the acylcarnitines back to the corresponding acyl-CoAs, the true substrates of the mFAO pathway (2–4). The sodium-driven, high-affinity organic cation/carnitine transporter 2 (OCTN2) provides the cell with the carnitine required for the transfer of acyl units (3, 4).

When mFAO is impaired, acyl-CoAs accumulate in the mitochondrial matrix. The accumulation of long-chain acyl-

Abbreviations: C8, octanoyl; C10, decanoyl; C10:0, decanoic acid; BSA, bovine serum albumin; CACT, carnitine/acylcarnitine translocase; CoA, coenzyme A; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; DMEM, Dulbecco's modified Eagle's medium; ESI-MS/MS, electrospray ionization tandem mass spectrometry; FBS, fetal bovine serum; IMM, inner mitochondrial membrane; L-AC, L-aminocarnitine; MCAD, medium-chain acyl-CoA dehydrogenase; MEM, minimum essential medium; mFAO, mitochondrial fatty acid β-oxidation; mFAOD, mitochondrial fatty acid β-oxidation disorder; OCTN2, organic cation/carnitine transporter 2; OMM, outer mitochondrial membrane; POCA, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate

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CoAs can have deleterious consequences, which include the disturbance of the oxidative phosphorylation system by inhibition of the dicarboxylate and ADP/ATP carriers (5–9). These intermediates, however, can be further metabolized by ω and ω-1 oxidation in the endoplasmic reticulum (leading to the production of dicarboxylic acids) or eliminated as acylglycines, acylcarnitines, or free acids. The most likely source for these acylcarnitines is the carnitine shuttle itself. It has been tentatively assumed that the carnitine shuttle is able to reverse its physiological action constituting a potential mitochondrial detoxification mechanism in case of a genetic or acquired mFAO disorder (mFAOD). This system would be responsible for the conversion of the acyl-CoAs, which accumulate within the mitochondrion into the respective acylcarnitines that would be further exported *via* the cytosol to the extracellular fluid. Although the plasma acylcarnitine intermediates are the most important biomarkers currently used in the postnatal diagnosis and neonatal screening of mFAODs (10–12), the true origin of these intermediates and the involvement of the carnitine shuttle in this process have never been experimentally verified thus far. The formation of acylcarnitines within the mitochondrion by the action of CPT2 has been previously shown by us (13, 14) and others (15) using *in vitro* models. However, the transport of these metabolites across the mitochondrial and plasmalemmal membranes into the extracellular space has remained undefined. To unambiguously define the true origin of the plasma acylcarnitine intermediates observed in patients with mFAODs, we developed a novel intact cell model. Using a lentiviral knockdown of the medium-chain acyl-CoA dehydrogenase (MCAD) in fibroblast cell lines of patients diagnosed with CPT2, CACT, or OCTN2 deficiency, we created double-knockdown cell lines. Subsequently, these cell lines were loaded with a medium-chain fatty acid that can enter the mitochondrion independently of the carnitine cycle. Additionally, we used a pharmacological approach and inhibited CPT1 and CPT2 in fibroblast cell lines from patients diagnosed with MCAD deficiency. These models not only mimic the accumulation of octanoylcarnitine (C8-carnitine), as seen in MCAD deficiency, but also allow the identification of the enzyme and transport proteins responsible for the postulated acylcarnitine export pathway.

MATERIALS AND METHODS

Materials

Dulbecco's modified Eagle's medium (DMEM), minimum essential medium (MEM), penicillin, streptomycin, and HEPES were obtained from Life Technologies (Invitrogen, Carlsbad, CA, USA). Fetal bovine serum (FBS) and L-glutamine were from BioWhittaker Lonza (Verviers, Belgium). Lipofectamine 2000, TRIzol, and the Superscript II Reverse Transcriptase kit were purchased from Invitrogen (Carlsbad, CA, USA). The LC480 SYBR Green I Master mix was from Roche (Mannheim, Germany). The D3-decanoic acid (C10:0) was from CDN Isotopes (Québec, QC, Canada). L-aminocarnitine (L-AC) was a gift from Sigma-Tau (Pomezia, Italy) and 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) was obtained from Byk Gulden Pharmazeutika (Konstanz, Germany). Puromycin, human serum albumin, bovine serum albumin (BSA), ferrocenium hexafluorophosphate, L-cysteine, L-carnitine, and acetylchloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cell culture conditions

Human skin fibroblasts were obtained from anonymized controls (n=4) and patients with established deficiencies at the level of CPT2 (n=1, p.[R124X];[R124X]), CACT (n=2, p.[G81R] homozygous on cDNA and c.[3G>A];[417+1 G>T]), OCTN2 (n=2, p.[P266R];[P266R] and p.[F23del];[F23del]) and MCAD (n=2 p.[K329E];[K329E] and p.[S245L];[S245L]).

HEK293 cells and primary skin fibroblasts were cultured in DMEM with 4.5 mg/ml glucose, 584 μg/ml L-glutamine, and 25 mM HEPES, supplemented with 10% FBS, 100 U/ml penicillin, 100 mg/ml streptomycin, and 100 mg/ml fungizone, in a humidified atmosphere of 5% CO_2, at 37°C.

Knockdown of MCAD in human fibroblasts using shRNA

MCAD shRNA-containing viruses were produced in HEK293 cells using Mission shRNA targeting the gene coding for MCAD (Sigma-Aldrich; TRCN0000028508 shRNA4; TRCN0000028564 shRNA5; TRCN0000028509 shRNA8). HEK293 cells were cotransfected with pMD2G, pMDL/RRE, pRSV/REV, and the shRNA TRC vector targeting MCAD using Lipofectamine 2000. After 24 h, the medium was refreshed. The next day, the virus-containing medium was collected, centrifuged, and stored at −80°C prior to transduction in order to prevent contamination with HEK293 cells. Primary skin fibroblasts from controls and CPT2-, CACT-, and OCTN2-deficient cell lines were incubated with the virus-containing medium. After 24 h, the medium was refreshed, and puromycin was added to a maximum concentration of 5 μg/ml to select for transduced cells.

Quantitative real-time PCR analysis

Total RNA was isolated from human fibroblasts with TRIzol, after which cDNA was prepared using the Superscript II reverse transcriptase kit. Quantitative real-time PCR analysis of selected genes was performed in this cDNA using the LC480 SYBR Green I Master mix. Primer sequences used were MCAD fw, 5′-AGAATTGGCTTATGGATGTACAGG-3′; MCAD rev, 5′-TTTGTTGATCATTTCCAGCAATAAT-3′; PPIB fw, 5′-TGGAGAGCACCAAGACAGACA-3′; PPIB rev, 5′-CTTCTCCACGATCTTGC-3′. To confirm the amplification of a single product, both melting curve and sequence analysis were carried out. All samples were analyzed in duplicate. Data were assessed using linear regression analysis, as described by Ramakers *et al.* (16). To compare the expression levels between different samples, values were normalized against cyclophillin (*PPIB*).

Determination of MCAD activity in human fibroblasts

For the determination of MCAD activity, fibroblast pellets were resuspended in PBS and homogenized by sonication (3 times, 10 s, 8 W). Protein concentration of the homogenates was determined using the bicinchoninic acid assay (17) and human serum albumin as a standard.

The standard mixture contained 250 mM Tris-HCl (pH 8.0) and 1.25 mM ferrocenium hexafluorophosphate plus 270 μM phenylpropionyl-CoA, in a final volume of 100 μl. The reaction was initiated by the addition of 80 μl of the standard mix to 20 μl of fibroblast homogenate (1 mg/ml final protein concentration) and was allowed to proceed at 37°C. After 10 min, the reaction was stopped with 10 μl of 2 M HCl, and samples were neutralized with 10 μl of a solution

containing 2 M KOH and 1 M MES (pH 6.0). After neutralization, 10 μl of 10 mM L-cysteine and 30 μl acetonitrile was added to the mixture. L-cysteine is added to reduce oxidized ferrocenium hexafluorophosphate, which may interfere with the chromatographic separation. The samples were centrifuged at 20,000 *g* for 5 min, and the metabolites in the supernatant were analyzed by HPLC with an elution system of acetonitrile and 16.9 mM sodium phosphate buffer pH 6.9.

Incubation of human fibroblasts with C10:0

Fibroblasts were plated in 48-well plates (~12.5 μg protein/well) and seeded overnight at 37°C. The following day, after washing the cells with PBS, the incubation mixture was added to each well and incubated in a humidified CO_2 incubator (5% CO_2, 95% air) at 37°C. Incubation mixtures contained MEM supplemented with BSA, essentially fatty acid free, 0.4 mM L-carnitine and 120 μM C10:0 to a final volume of 250 μl. For the inhibition studies, POCA and L-AC were added to the incubation mixture to a final concentration of 3 μM and between 0 and 2000 μM, respectively. After 72 h, the medium was collected, and the cells were washed and resuspended in 100 μl of PBS with 1 mg/ml Triton X-100 to measure protein content. To 50 μl medium, 560 μl acetonitrile was added containing 50 pmol of D3-propionylcarnitine (C3) and 20 pmol D3-palmitoylcarnitine (C16) internal standards. After derivatization of the produced acylcarnitines with 1-butanol/acetylchloride 4/1 (v/v), these intermediates were quantified by electrospray ionization tandem mass spectrometry (ESI-MS/MS) essentially as described previously (18).

RESULTS

Analysis of the MCAD knockdown efficiency in human fibroblasts

We first determined the knockdown efficiency of five different MCAD shRNAs in control fibroblasts by evaluating the gene expression at the mRNA level by quantitative real-time PCR. The MCAD shRNA4 and MCAD shRNA5 showed the highest knockdown efficiency (**Fig. 1**), and a mixture of these two shRNAs was chosen for subsequent experiments in control and CPT2-, CACT-, and OCTN2-deficient fibroblasts. Knockdown was further assessed by measuring MCAD activity in both control and deficient cell lines (**Fig. 2**). The efficiency of the MCAD shRNAs varied between cell lines, with 44 to 70% reduction of the enzyme activity relative to the respective nontarget shRNA cell line.

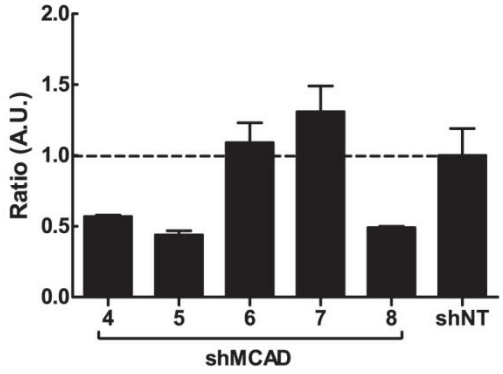

Figure 1. Evaluation of the knockdown efficiency of different MCAD shRNAs (shMCAD 4 to 8) in control fibroblasts. MCAD gene expression was measured at the mRNA level by quantitative real time PCR. For experimental details. see Materials and Methods. Expression level of the nontarget shRNA (shNT) was set to 1. Results are represented in arbitrary units (A.U.) by the ratio between the expression levels of each sample and cyclophillin.

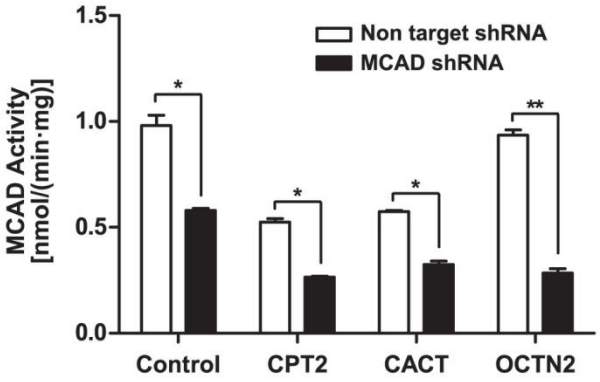

Figure 2. Evaluation of the MCAD knockdown efficiency in human fibroblasts from control subjects and patients with CPT2, CACT, or OCTN2 deficiency. After transfection with the nontarget shRNA (negative control) or with the MCAD shRNA, MCAD activity was determined by HPLC. For experimental details, see Materials and Methods. Data are expressed as means ± SD of duplicates of ≥2 independent experiments. $*P < 0.05$, $**P < 0.001$.

Acylcarnitine analysis in control fibroblasts after MCAD knockdown

Control fibroblast cell lines with and without the MCAD knockdown were incubated with C10:0, a medium-chain fatty acid. Medium-chain fatty acids are able to cross the cellular and mitochondrial membranes by diffusion. Within the mitochondrion, they are activated to their respective CoA ester. Decanoyl-CoA (C10-CoA) will undergo one β-oxidation cycle, leading to the formation of octanoyl-CoA (C8-CoA). Because of the MCAD knockdown, further mitochondrial β-oxidation of C8-CoA will be impaired at this point. As a consequence, this intermediate will accumulate in the matrix, followed by export to the external medium in the form of C8-carnitine. Indeed, increased levels of C8-carnitine (3-fold, $P<0.01$) were detected in the extracellular medium of control fibroblasts after MCAD knockdown (**Fig. 3**), which validates our model.

Acylcarnitine profiles in CPT2-, CACT-, and OCTN2-deficient fibroblasts after MCAD knockdown

CPT2-, CACT- and OCTN2-deficient fibroblast cell lines, with and without the MCAD knockdown, were incubated with C10:0 fatty acid. In the case of CPT2- and CACT-deficient cell lines with MCAD knockdown, the levels of C8-carnitine observed in the extracellular medium were comparable with the respective negative control (Fig. 3). These results suggest that CPT2 and CACT are crucial for the mitochondrial formation and export of C8-carnitine. Surprisingly, while in CPT2-

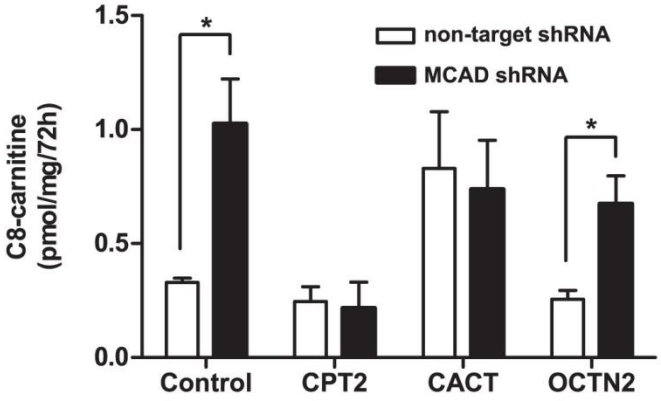

Figure 3. Levels of C8-carnitine present in the extracellular medium of human fibroblasts (controls and CPT2-, CACT-, and OCTN2-deficient cell lines with and without MCAD knockdown). Acylcarnitines were determined in the extracellular medium after incubation of the human fibroblast cell lines with C10:0 and L-carnitine and quantified by ESI-MS/MS. For experimental details, see Materials and Methods. Data are means ± SD of duplicates of ≥2 independent experiments. All experiments were carried out with 2 independent cell lines, except in the case of CPT2 deficiency, in which only one cell line was used.*$P < 0.01$.

deficient cells C8-carnitine values were low, in the case of CACT deficiency, the levels of C8-carnitine increase in equal manner in the cells with and without MCAD knockdown, when compared with control cells, suggesting an extramitochondrial mechanism for the production of these acylcarnitines.

As OCTN2 is the high-affinity plasma carnitine transporter, we hypothesized that it might also play a role in the cellular export of acylcarnitines. However, in contrast to CPT2- and CACT-deficient cells, C8-carnitine levels in the extracellular medium of OCTN2-deficient cells were higher (2.8-fold, $P<0.01$) after MCAD knockdown when compared to OCTN2-deficient cells without MCAD knockdown, which served as a negative control (Fig. 3). This provides evidence against the involvement of OCTN2 in the transport of acylcarnitines across the cell membrane.

Acylcarnitine profiles in MCAD-deficient fibroblasts after pharmacological inhibition of CPT1 and CPT2

To validate the results obtained with the double-knockdown models, it was important to confirm that decanoic acid enters the mitochondrion by diffusion and, thus, independently of the carnitine shuttle. For this purpose, we used POCA, an irreversible inhibitor of CPT1 activity, which blocks the conversion of acyl-CoAs into acylcarnitines and therefore limits the access of fatty acids to the matrix *via* the carnitine shuttle. We incubated MCAD-deficient cells with C10:0 in the presence or absence of 3 μM of POCA, which gives nearly complete CPT1 inhibition, and increasing concentrations of the CPT2 inhibitor L-AC (19, 20). The POCA concentration used in these experiments was selected by monitoring the levels of C8-carnitine in the medium of MCAD-deficient cells incubated with C16:0, the preferred CPT1 substrate, and increasing concentrations of POCA (results not shown). We found that POCA, in the absence of L-AC, decreases the levels of C8-carnitine in the medium by 50%, indicating that C10:0 enters the mitochondrion partly *via* the carnitine shuttle, but more important, also by diffusion (**Fig. 4**). The C8-carnitine produced after inhibition of CPT1 in MCAD-deficient cells incubated with C10:0 can be dose dependently reduced in the presence of L-AC (Fig. 4). This decrease observed in extracellular C8-carnitine levels with increasing inhibition of CPT2 confirms the crucial role of this enzyme in the mitochondrial synthesis of acylcarnitines.

DISCUSSION

Here, we developed a novel intact cell model to address the true origin of the acylcarnitine species that are detected in plasma from mFAO-deficient patients. With this model, we investigated the involvement of CPT2, CACT, and OCTN2 in the synthesis and export of acylcarnitines out of the mitochondrion and subsequently out of the cell. For this, we created double-knockdown cell lines by silencing MCAD expression in fibroblast cell lines of patients diagnosed with CPT2, CACT, or OCTN2 deficiency. Subsequently, these cell lines were loaded with C10:0 fatty acid since the entry of medium-chain fatty acids into the mitochondrion is believed to occur *via* diffusion and independently of the carnitine cycle (21–23). This system allowed us to study the role of CPT2, CACT, and OCTN2 in the formation and export of C8-carnitine. In addition, we used a pharmacological approach in MCAD-deficient cell lines to inhibit CPT1 and CPT2 with POCA and L-AC, respectively. These cells were incubated with C10:0 to induce the formation and export of C8-carnitine. Using this approach, we could address the role of CPT2 in the mitochondrial synthesis of acylcarnitines. A similar pharmacological approach for CACT and OCTN2 was not possible since specific inhibitors of these transporters are not available.

The inhibition of CPT1 in the MCAD-deficient cell lines indicated that C10:0 also uses CPT1 (and thus the

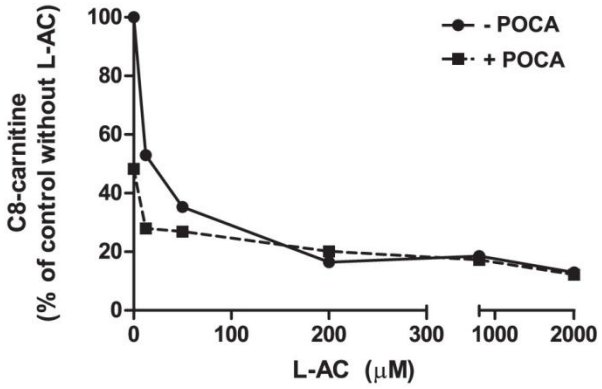

Figure 4. Levels of C8-carnitine present in the extracellular medium of MCAD-deficient cell lines after incubation with C10:0 in the presence or absence of 3 μM of the CPT1 inhibitor POCA and increasing concentrations of the CPT2 inhibitor L-AC.

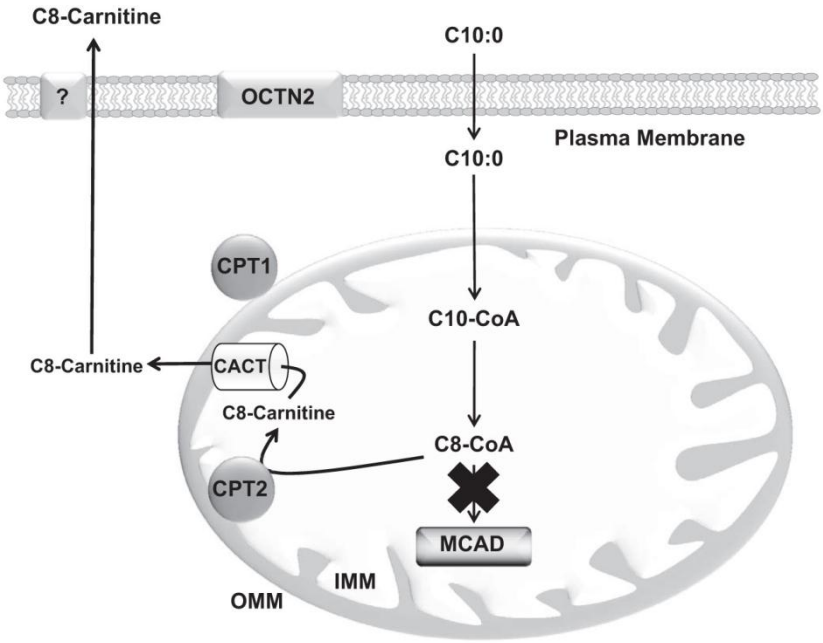

Figure 5. Schematic representation of the proposed catabolism of medium-chain fatty acids (C10:0) in cells with MCAD knockdown. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; C8, octanoyl; C10, decanoyl.

carnitine shuttle) to access the mitochondrial matrix. However, as evidenced by the elevated levels of C8-carnitine in the medium after CPT1 inhibition, ≥50% of the C10:0 is able to enter the mitochondrion by diffusion, validating the results obtained with the double-knockdown models.

The reversibility of the CPT2 enzyme reaction has been verified experimentally by *in vitro* studies (13–15). We have recently established the substrate specificity of CPT2 and demonstrated its ability to convert saturated and unsaturated medium- and long-chain acyl-CoA esters into the corresponding acylcarnitine esters (14). In the present work, we aimed to address the relevance of these findings in an intact cell model. Using the genetic and pharmacological approach introduced above, we demonstrate that CPT2 is the enzyme responsible for the mitochondrial conversion of C8-CoA into C8-carnitine (**Fig. 5**).

We also studied the role of CACT in the mitochondrial export mechanism of acylcarnitines. Under normal conditions, this transporter is responsible for the import of acylcarnitines into the mitochondrial matrix. The results in our double-knockdown model reveal no differential effect between MCAD knockdown cells and the respective negative control (Fig. 3), indicating that the mitochondrial export of acylcarnitines is compromised in CACT deficiency (Fig. 5). These results are in agreement with previous data reported by Palmieri and colleagues (24) using recombinant CACT expressed in *Escherichia coli* and reconstituted into liposomes. In this *in vitro* model, the uptake of radiolabeled carnitine into proteoliposomes preloaded with different substrates was measured, and the reconstituted transporter showed affinity for short-, medium-, and long-chain acylcarnitines. Although the reconstituted transporter is believed to be oriented as in the native membrane (25), the true orientation of CACT within an artificial bilayer has never been experimentally demonstrated so far. Because our model employs intact cells, we can now conclude that CACT is able to export medium-chain acylcarnitines. Despite a clear function of CACT in the transport of acylcarnitines across the IMM, the mechanism by which these metabolites are transported across the outer mitochondrial membrane (OMM) to the cytosol remains unidentified. The voltage-dependent anion channel (VDAC) is a likely candidate to mediate the passage of acylcarnitines through the OMM, but further studies are required to address this issue.

Likewise, the mechanism of cellular export of the acylcarnitine intermediates has remained unresolved. We hypothesized that OCTN2 could have a role in this process by facilitating the transport of acylcarnitine molecules into the extracellular medium. OCTN2 is the protein responsible for the plasmalemmal sodium-dependent import of carnitine (26, 27), and it has also been reported to transport short-chain acylcarnitines like acetyl- and propionylcarnitine (28). Interestingly, it has been shown that OCTN2 may also function as an antiporter (29, 30). Indeed, external sodium cotransport with carnitine is coupled to antiport of carnitine, acylcarnitines, and other substrates. This antiport is functionally asymmetrical as the internal K_m for carnitine is one order of magnitude higher than the external K_m (30). We show here that even without a functional OCTN2, medium-chain acylcarnitines are able to cross the plasma membrane (Fig. 3), demonstrating that this transporter is not essential in the cellular export of these intermediates (Fig. 5). Nevertheless, we cannot exclude that OCTN2 may be partially responsible for this mechanism and/or that another transport protein allows acylcarnitine export when OCTN2 is not functional. Other transporters in the plasma membrane, such as the ones associated with the cellular uptake of free fatty acids, including the fatty acid binding protein (FABPpm), fatty acid transport protein (FATP), and fatty acid translocase (FAT/CD36) may also need to be taken in consideration as potential players in the cellular export of acylcarnitines as fatty acyl derivatives.

In summary, we have demonstrated that in the case of the mitochondrial accumulation of medium-chain acyl-CoAs as

a result of a genetic deficiency within mFAO, these potentially toxic intermediates are converted into the respective acylcarnitines *via* CPT2 and further transported across the inner mitochondrial membrane through CACT. We could not evaluate the export of long-chain acylcarnitines in our model as the different components of the carnitine shuttle, essential for the transport of long-chain acyl units across the mitochondrial membrane system are deficient and would not allow the entry of long-chain fatty acids into mitochondria. Given the results herein obtained and the role of CPT2 and CACT in the mitochondrial import of long-chain acyl units, we suggest that these are as well major players in the mitochondrial synthesis and export of long-chain acylcarnitines. The identity of the plasmalemmal exporter of acylcarnitines remains unclear, but our results indicate that OCTN2 is not involved (or at least not exclusively) in the cellular export of these metabolites. FJ

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Chapter 6

Carnitine palmitoyltransferase 1A is not crucial for the translocation of acylcarnitines across the outer mitochondrial membrane

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Carnitine palmitoyltransferase 1A is not crucial for the translocation of acylcarnitines across the outer mitochondrial membrane

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Abstract

Carnitine palmitoyltransferase 1 (CPT1) is an integral protein of the outer mitochondrial membrane (OMM) that catalyzes the conversion of long-chain acyl-CoAs into the respective carnitine esters, the rate limiting reaction in long-chain fatty acid β-oxidation. It is unclear how these acylcarnitine intermediates further cross the OMM to be transported into the mitochondrial matrix for β-oxidation. Rat CPT1A has been claimed to form a hexameric quaternary structure that was hypothesized to be involved in the channelling of acylcarnitines. We aimed to investigate the potential role of CPT1A in the transport of acylcarnitines into the intermembrane space and its dependence on the transferase activity. Therefore, we studied the oxidation of palmitoylcarnitine in isolated mitochondria from human skin fibroblasts of controls and of an individual that expresses no CPT1A protein. In addition, we assessed oxidation of pristanic acid in intact cells, as this pathway requires the transfer of acylcarnitines from peroxisomes to mitochondria. We found that the oxidation of both substrates is similar between control and the CPT1A-deficient cell line. In conclusion, even in the absence of CPT1A protein, acylcarnitines are able to access the mitochondrial matrix, arguing against a crucial role for CPT1A in the channelling of these intermediates.

Keywords: Mitochondria; Outer mitochondrial membrane; Fatty acid oxidation; Acylcarnitine channelling

Introduction

Carnitine palmitoyltransferase 1 (CPT1) is a member of the carnitine acyltransferase family. Localized in the outer mitochondrial membrane (OMM), this transmembrane protein catalyzes the rate-limiting reaction of the mitochondrial long-chain fatty acid β-oxidation pathway (mFAO). mFAO is particularly important during prolonged fasting or physical exercise, providing tissues such as the liver, heart and skeletal muscle with fatty acids that can be used to generate energy [1, 2].

In order to be oxidized within the mitochondria, long-chain fatty acids must first undergo activation to acyl-coenzyme A (CoA) by a long-chain acyl-CoA synthetase and then further converted into the respective carnitine ester in a process catalysed by CPT1. There are three known isoforms of this enzyme, encoded by different genes and with different tissue specificities: CPT1A, which is expressed ubiquitously with high levels in liver and kidney; CPT1B, which is mainly present in cardiac and skeletal muscle; and CPT1C which is expressed in brain [3, 4].

The localization and topology of CPT1 in the membrane has been subject of discussion for decades and still remains a controversial topic. The first studies localized the CPT1 activity to the outer

Abbreviations: CoA, coenzyme A; CACT, carnitine/acylcarnitine translocase; CPT1A, carnitine palmitoyltransferase 1A; CPT2, carnitine palmitoyltransferase 2; CrAT, carnitine acetyltransferase; CrOT, carnitine octanoyltransferase; DMN, dimethylnonanoyl; IMM, inner mitochondrial membrane; IMS, intermembrane space; mFAO, mitochondrial fatty acid β-oxidation: OMM, outer mitochondrial membrane; VDAC, voltage dependent anion channel.

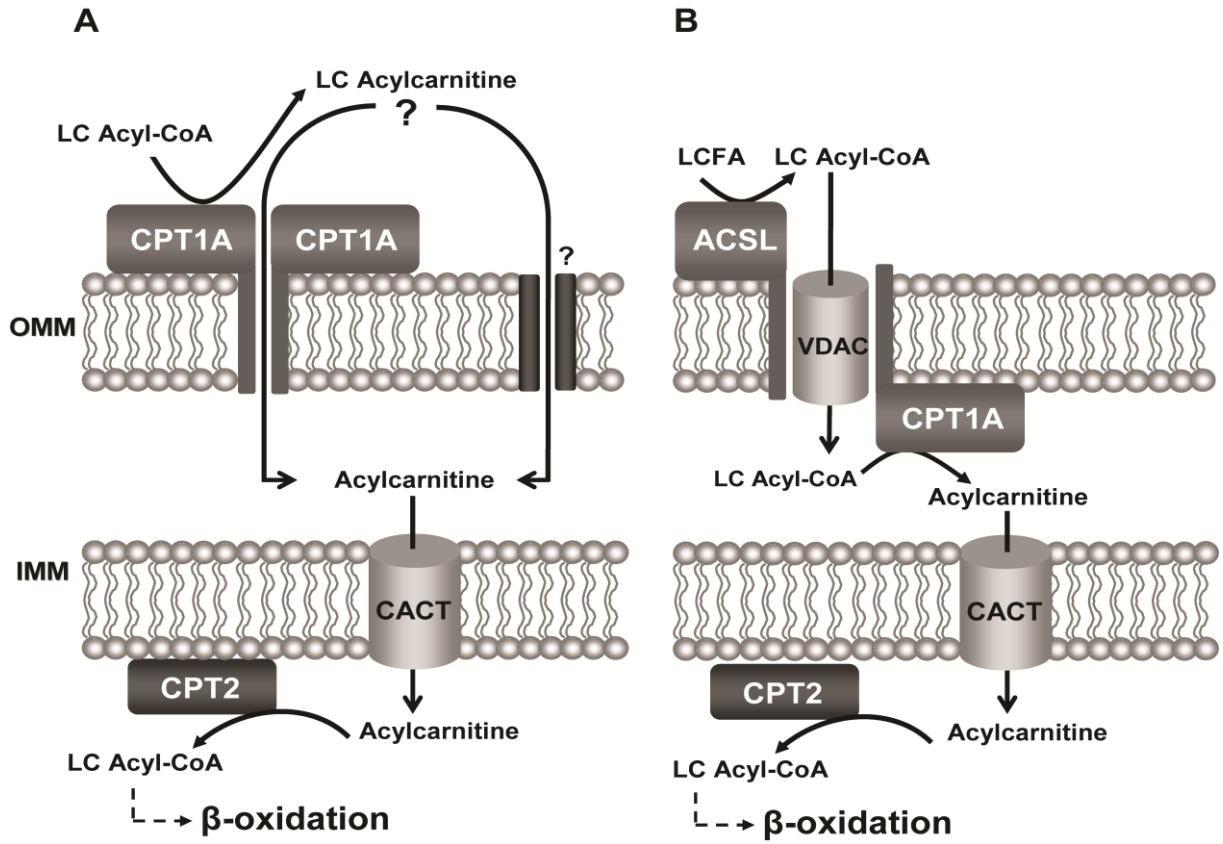

Fig. 1. Schematic representation of the proposed mechanism of (A) passage of long-chain acylcarnitines formed in the cytosol by CPT1A across the outer mitochondrial membrane (OMM) via the putative pore formed by CPT1A oligomerization and (B) the inverted model of CPT1A (adapted from Lee et al. [12]), where long-chain acyl-CoAs cross the OMM through the voltage dependent anion channel (VDAC) before being converted, within the mitochondrial intermembrane space, by CPT1A into the corresponding acylcarnitine ester. ACSL, long-chain acyl-CoA synthetase; CACT, carnitine/acylcarnitine translocase; CPT1, carnitine palmitoyltransferase 1; CPT2, carnitine palmitoyltransferase 2; IMM, inner mitochondrial membrane; LC, long-chain; LCFA, long-chain fatty acid.

aspect of the inner mitochondrial membrane (IMM) [5-7] and only later this protein was assigned to the OMM with the regulatory and catalytic domains believed to be located on opposite sides of the membrane [8]. Ten years later, Fraser and co-workers [9] reported convincing experiments suggesting a bitopic topology of CPT1 in the OMM, with two transmembrane domains connected by a loop protruding to the intermembrane space (IMS) and both catalytic and regulatory domains (C- and N-terminal, respectively) facing the cytosol (for review see [10]). Recently, the debate on CPT1A has extended from its orientation and topology in the membrane [11, 12] to a possible arrangement as an oligomeric structure. It was shown that rat CPT1A forms an oligomeric complex in the OMM [13] and that the transmembrane domain 2 has a prominent role in this oligomerization [14]. This would lead to a quaternary structure arranged as a trimer. Two trimers were proposed to associate as a dimer forming a hexamer, which resembles a pore-like structure within the OMM [13, 14].

Due to the proposed cytosolic orientation of the CPT1 catalytic site, the general idea is that acylcarnitines are formed in the cytosol. However, it remains unclear how these intermediates have access to the IMS to be further transported by the carnitine/acylcarnitine translocase (CACT) to the mitochondrial matrix [15]. Once in the matrix, the long-chain acylcarnitines are finally converted by carnitine palmitoyltransferase 2 (CPT2) back to the initial long-chain acyl-CoAs, the true substrates for mFAO. Based on the potential oligomerization of CPT1A, it has been suggested that the enzyme not only catalyses the formation of acylcarnitines, but may also facilitate the channelling of these derivatives formed in the cytosol into the IMS (Fig.

1A) [13, 14]. Nevertheless, this hypothesis has never been experimentally tested so far.
It has been also hypothesised that acylcarnitines are formed in the IMS [8]. This subject was recently readdressed by Lee and co-workers [12] where these authors suggest that the catalytic site of CPT1 faces the IMS, arguing against the proposed topology of the enzyme [9]. Such orientation in the membrane would, as a first step, require the translocation of the large acyl-CoA units through the OMM. The voltage dependent anion channel (VDAC) is the proposed candidate to facilitate the transport of acyl-CoA units (Fig. 1B) as it has been demonstrated previously that its inhibition affects the oxidation of palmitoyl-CoA but does not seem to interfere with the oxidation of palmitoylcarnitine [16]. In this paper we aimed to investigate if CPT1A is involved in the pathway by which acylcarnitines access the IMS. Using different acylcarnitine intermediates and a patient cell line carrying a nonsense mutation in the *CPT1A* gene that prevents the expression of a functional protein, we show that CPT1A is not crucial for the mitochondrial uptake of acylcarnitines and that another mechanism must exist by which acylcarnitines cross the OMM.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), HAM medium, penicillin, streptomycin, fungizone and Hepes were obtained from Gibco Invitrogen (Carlsbad, California, USA). Fetal bovine serum (FBS) and L-glutamine were from BioWhittaker Lonza (Verviers, Belgium). Trizol and the Superscript II Reverse Transcriptase kit were purchased from Invitrogen (Carlsbad, California, USA). The LC480 Sybr Green I Master mix was from Roche (Mannheim, Germany). Isotope labeled [U-^{13}C]-palmitoyl-CoA was chemically synthetized as described by Rasmussen *et al* [17] and [U-^{13}C]-palmitoylcarnitine was obtained from Isotec, Sigma-Aldrich (St. Louis, MO, USA). Radiolabeled [1-^{14}C] pristanic acid was synthesized as described by Singh *et al* [18], unlabeled pristanic acid and the internal standards (d3-C3-, d3-C8 and d3-C16-carnitine) were synthesized by Herman ten Brink (VU Medical Center, Amsterdam, The Netherlands). Human serum albumin (HSA), bovine serum albumin (BSA), L-carnitine and acetylchloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cell culture conditions

Human skin fibroblasts were obtained from anonymized controls (n=4) and patients with established deficiencies at the level of CPT1A (p.[E525GfsX32];[E525GfsX32]), CPT2 (p.[R124X];[R124X]) and CACT (c.[3G>A];[417+1 G>T]). Primary skin fibroblasts were cultured at 37ºC, in a humidified atmosphere of 5% CO_2, in DMEM with 4.5 g/L glucose, 584 mg/L L-glutamine and 25 mM Hepes, supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin and 100 mg/mL fungizone.

Quantitative real-time PCR analysis

Total RNA was isolated from human skin fibroblasts with Trizol, after which cDNA was prepared using the Superscript II Reverse Transcriptase kit. Quantitative real-time PCR analysis (qPCR) of selected genes was performed in this cDNA using the LC480 Sybr Green I Master mix. Primer sequences are available upon request. To confirm the amplification of a single product, the samples were analysed on an agarose gel and a melting curve was performed. The identity of the amplicon was confirmed by sequence analysis. All samples were analyzed in duplicate. Data was assessed using linear regression analysis as described by Ramakers *et al* [19]. To compare the expression levels between different samples, values were normalized against Cyclophillin (*PPIB*).

Isolation of mitochondria from human skin fibroblasts

After reaching confluence, fibroblasts were harvested by trypsinization and washed twice with phosphate buffer saline (PBS). The pellet was resuspended in 1 mL ice-cold SEM buffer (250 mM sucrose, 1 mM EDTA and 5 mM MOPS buffer, pH 7.4) and homogenized at 4ºC by passing 15 times through a cell cracker device (EMBO, Germany) with a 6 μm gap. The suspension was centrifuged at 400*g* for 5 min at 4ºC and the supernatant, corresponding to the total organellar fraction, was collected in a new tube. This procedure was repeated once with the pellet and the collected supernatant was

centrifuged at 4020*g* for 10 min at 4ºC in order to obtain the mitochondrial fraction. The mitochondrial pellet was resuspended in 1 mL ice-cold SEM and was used to measure palmitoylcarnitine oxidation. Protein content of the mitochondrial fraction was determined using the bicinchoninic acid assay [20] and HSA as standard.

Palmitoylcarnitine oxidation in isolated mitochondria from control and CPT1A-deficient human skin fibroblasts

The reaction mixture (final volume 250 µL) consisted of SEM buffer, 2 mM EDTA, 20 mM potassium phosphate, 1 mg/mL BSA, 4.5 mM reduced glutathione, 0.5 mM L-carnitine and 25 µM [U-^{13}C]-palmitoyl-CoA or 25 µM [U-^{13}C]-palmitoylcarnitine, pH 7.4. Reactions were started by adding 50 µL of the mitochondrial suspension (final protein concentration of 0.1 mg/mL) to the reaction mixture, followed by an incubation period of 10 min at 37ºC. To stop the reaction, 800 µL of acetonitrile were added containing 100 pmol of d3-propionylcarnitine (C3), 100 pmol of d3-octanoylcarnitine (C8) and 300 pmol d3-palmitoylcarnitine (C16) as internal standards. Subsequently, the reaction mixtures were centrifuged at 20,000*g* for 5 min at 4°C and the supernatant collected and dried under a nitrogen stream. After derivatization of the produced acyl-carnitines with 100 µL of 1-butanol:acetylchloride 4:1 (v/v), these intermediates were quantified by Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS) essentially as previously described [21].

Citrate synthase activity measurement

The levels of acetylcarnitine formed upon the oxidation of palmitoylcarnitine in isolated mitochondria were normalized for mitochondrial content, which is a better parameter than protein content alone. In order to do this, we measured the activity of citrate synthase, an enzyme only present in the mitochondrial matrix and which activity is expected to be correlated with the mitochondrial content. Citrate synthase activity was measured using the DTNB (5,5'-dithiobis-(2-nitrobenzoic acid) based assay as described before [22]. The procedure was carried out on a TECAN Freedom EVO robot equipped with an Infinite plate reader.

Acylcarnitine profiling of pristanic acid oxidation in intact human skin fibroblasts

The acylcarnitine profiling in fibroblasts from controls and CPT1A-, CPT2- and CACT-deficient patients was performed essentially as described before by Ventura *et al* [23] with minor modifications. Incubation mixtures contained MEM supplemented with 4 mg/mL BSA essentially fatty acid free, 0.4 mM L-carnitine and 120 µM pristanic acid to a final volume of 1 mL. After 72h incubation, 50 µL of medium was collected and mixed with 560 µL acetonitrile containing internal standards (50 pmol of d3-C3-carnitine, 50 pmol of d3-C8-carnitine and 20 pmol of d3-C16-carnitine). Derivatization and analysis of the produced acylcarnitines were performed as described above. The cells were washed and resuspended in 400 µL of PBS with 1 g/L Triton X-100 to measure protein content using the bicinchoninic acid assay [20] and HSA as standard.

Oxidation of [1-^{14}C]-pristanic acid in intact human skin fibroblasts

Upon incubation of intact fibroblasts with pristanic acid, propionyl-CoA is formed in the first cycle of the peroxisomal β-oxidation and is subsequently converted to a carnitine ester by peroxisomal carnitine acetyltransferase (CrAT). The resultant propionylcarnitine, formed within the peroxisome, will be imported into the mitochondria and metabolized to CO_2 [24]. The total peroxisomal and mitochondrial β-oxidation activities are quantified as the sum of the amount of $^{14}CO_2$ released and the amount of [1-^{14}C]-acid soluble products (ASP) formed, as described before [25]. The mitochondrial oxidation of propionylcarnitine to CO_2 is normalized for the total amount of labelled species produced in order to correct for the peroxisomal β-oxidation by calculating the ratio $CO_2/(CO_2 + ASP)$.

Oxidation of radioactive [1-^{14}C]-pristanic acid was measured in human control, CPT1A- and CACT-deficient fibroblasts essentially as described before [24] with minor modifications. The standard mixture (final volume 250 µL) contained 5 µM of radiolabeled [1-^{14}C]-pristanic acid, 20 µM of unlabeled pristanic acid, 1 mg/mL β-cyclodextrine, 2 mM L-carnitine and HAM medium without FBS to a final volume of 500 µL. The reaction was initiated by the addition of the standard mixture to each

reaction vial and was allowed to proceed at 37ºC. After 2 hours incubation, the reaction was stopped with 100 µL of 2.6 M perchloric acid and radioactivity was measured in a β-counter (Packard 1600 CA/2000/2900).

Results

Analysis of CPT1A mRNA expression in control and CPT1A-deficient human skin fibroblasts

The CPT1A-deficient cell line used for this study has a homozygous c.1573dup mutation resulting in a frameshift and a premature stop codon 31 amino acid residues downstream (p.E525GfsX32). A premature stop codon upstream of the last exon often results in an unstable mRNA which is degraded in the cell via a pathway known as nonsense-mediated decay [26], likely resulting in the absence of the mutant protein from the deficient cell line. In order to determine whether the CPT1A protein is present in the deficient fibroblasts we evaluated its expression at the mRNA level. CPT1A mRNA levels in the CPT1A-deficient cell line were very low compared with the control fibroblasts (Fig. 2). These results show that *CPT1A* expression is virtually absent in the selected CPT1A-deficient cell line.

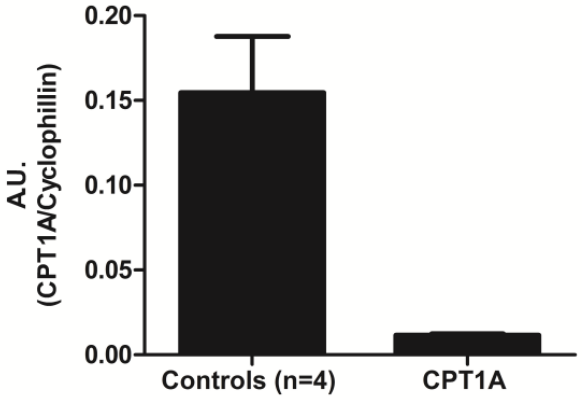

Fig. 2. Evaluation of *CPT1A* gene expression at the mRNA level by quantitative real time PCR in controls (4 cell lines) and CPT1A deficient human skin fibroblasts (1 cell line). Data shown is normalized against Cyclophillin (*PPIB*) and is the mean ± SD of triplicate samples. For experimental details see Materials and Methods.

Palmitoylcarnitine oxidation in isolated mitochondria from control and CPT1A deficient human skin fibroblasts

Isolated mitochondria from control and CPT1A-deficient human skin fibroblasts were incubated with isotope labelled [U-^{13}C]-palmitoylcarnitine (C16-carnitine) or [U-^{13}C]-palmitoyl-CoA (C16-CoA). Oxidation of these substrates will yield [U-^{13}C]-acetyl-CoA. In the absence of ADP, further oxidation of acetyl-CoA in the Krebs cycle will be slow and due to the excess of carnitine most of the acetyl-CoA will be converted into acetylcarnitine by the mitochondrial CrAT. We used the accumulation of acetylcarnitine to quantify the oxidation of the fatty acyl substrates.

In control cell lines, C16-CoA is oxidized and high levels of acetylcarnitine were found in the extracellular medium. This oxidation is inhibited by the addition of malonyl-CoA, the physiological inhibitor of CPT1. In CPT1A-deficient cells oxidation of C16-CoA is, as expected, severely impaired which is evidenced by the low levels of acetylcarnitine produced (Fig. 3).

When C16-carnitine is used as a substrate instead, the transferase reaction catalyzed by CPT1A is bypassed, but a potential role of CPT1A in the channelling of this acylcarnitine into the IMS would still be necessary. The rate of oxidation of C16-carnitine in the CPT1A-deficient fibroblasts was not significantly different from controls (Fig. 3). This indicates that CPT1A is not obligatory for the oxidation of palmitoylcarnitine and that the long-chain acylcarnitine is able to enter the mitochondria for oxidation. Interestingly, in the presence of malonyl-CoA the oxidation of palmitoylcarnitine was significantly reduced in control cell lines, but not in CPT1A-deficient fibroblasts (Fig. 3). These results suggest that CPT1A plays a role in the metabolism of C16-carnitine, but is unlikely to participate directly in the channelling of long-chain acylcarnitines across the OMM.

Acylcarnitine profiles of control and CPT1A-, CPT2- and CACT-deficient human skin fibroblasts upon incubation with pristanic acid

Pristanic acid (2,6,10,14-tetramethylpentadecanoic acid) is a long branched-chain fatty acid (C19), which is initially metabolized in the peroxisome. Three cycles of pristanic acid β-oxidation in

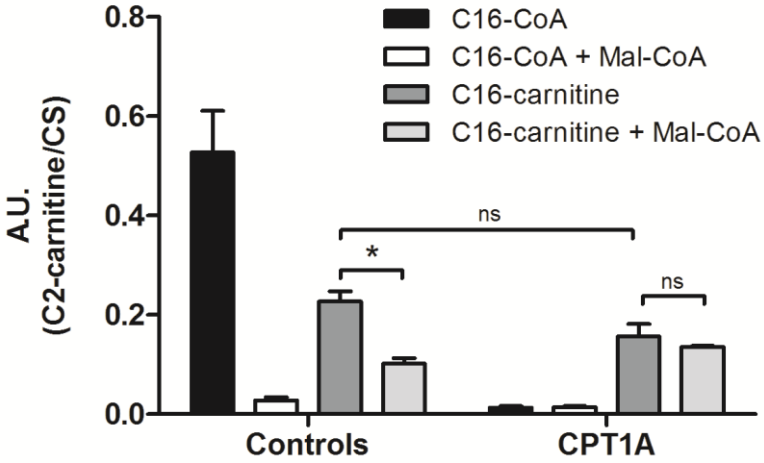

Fig. 3. Incubation of mitochondria isolated from control (4 cell lines) and CPT1A deficient fibroblasts (1 cell line) with palmitoyl-CoA (C16-CoA), palmitoyl-CoA and malonyl-CoA (C16-CoA + Mal-CoA), palmitoylcarnitine (C16-carnitine) and palmitoylcarnitine and malonyl-CoA (C16-carnitine + Mal-CoA). Values are represented as arbitrary units (A.U.) and correspond to the level of [^{13}C]-acetylcarnitine (C2-carnitine) normalized for mitochondrial content by measuring citrate synthase (CS) activity. Data shown are the mean ± SD of two independent experiments performed in duplicate. For experimental details see Materials and Methods. Significantly different values are marked with one asterisk (*p* value < 0.01). The symbol *ns* denotes a non-significant difference.

peroxisomes yield a branched-chain intermediate with eleven carbon atoms (C11), dimethylnonanoyl-CoA (DMN-CoA). After conversion to the respective carnitine ester within the peroxisome, presumably by carnitine octanoyltransferase (CrOT), this metabolite will be transported to the mitochondria for further oxidation [27]. In control cells loaded with pristanic acid no accumulation of DMN-carnitine was observed, which indicates proper mitochondrial uptake and degradation of this intermediate. As a positive control, cells with a deficiency at the level of CACT or CPT2 accumulate extracellular DMN-carnitine indicating these cells are not able to import or degrade this carnitine ester [27, 28]. In the case of CPT1A-deficient fibroblasts, and similarly to control cells, no DMN-carnitine accumulation was observed in the external medium (Fig. 4). This indicates that CPT1A does not take part in the mitochondrial uptake of this branched medium-chain acylcarnitine intermediate.

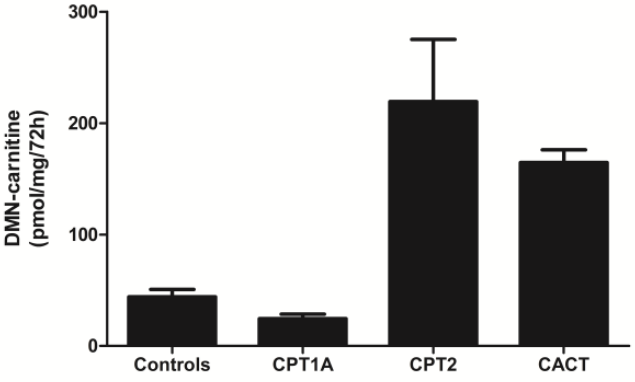

Fig. 4. Levels of dimethylnonanoylcarnitine (DMN-carnitine), product of peroxisomal pristanic acid oxidation, measured in the external medium of control (2 cell lines) and CPT2-, CACT- and CPT1A-deficient human skin fibroblasts (1 cell line per deficiency). Data shown are the mean ± SD of two independent experiments performed at least in duplicate. For experimental details see Materials and Methods.

Oxidation of [1-^{14}C]-pristanic acid in control and in CPT1A- and CACT-deficient human skin fibroblasts

In the first and third cycle of the peroxisomal β-oxidation of pristanic acid, the released propionyl-CoA is subsequently converted to a carnitine ester by peroxisomal CrAT and transported to the mitochondria where it is oxidized to CO_2. By making use of [1-^{14}C]-pristanic acid the mitochondrial import of propionylcarnitine can be studied in an intact cell system. The mitochondrial oxidation of propionylcarnitine was similar in control and CPT1A-deficient fibroblasts (Fig. 5). In a CACT-deficient cell line (used as a negative control), propionylcarnitine is not able to enter the mitochondria and thus CO_2 production is reduced.

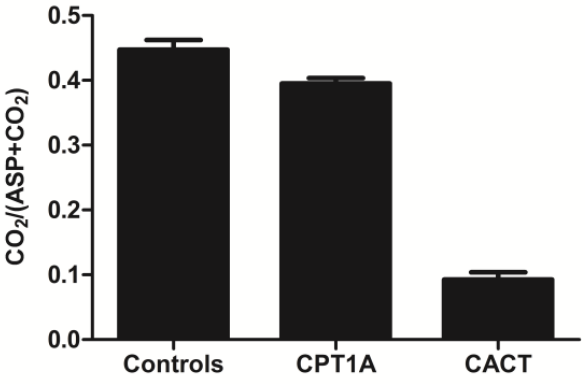

Fig. 5. Evaluation of the oxidation of [1-^{14}C]-pristanic acid in control (3 cell lines) and in CACT- and CPT1A-deficient fibroblasts (1 cell line per deficiency). In order to normalize for the peroxisomal β-oxidation, values are expressed as the ratio between the produced $^{14}CO_2$ and the total amount of products formed (CO_2 + ASP). Data shown are the mean ± SD of two independent experiments performed at least in duplicate. ASP: acid soluble products. For experimental details see Materials and Methods.

This shows that in contrast to CACT, CPT1A is not required for the entry of propionylcarnitine into the mitochondria for further oxidation.

Discussion

Although CPT1 has been studied for decades, its function and structural features have been the subject of discussion until today. Especially in the last years, new hypotheses have been brought forward concerning the oligomerization status of the enzyme and its topology in the membrane. Recent studies point to the existence of a hexameric protein structure with an unknown function. It has been hypothesised that this hexamer would form a pore, assigning to CPT1A a role in the passage of acylcarnitines across the OMM [13, 14], besides its activity in the formation of these derivatives. To experimentally test this hypothesis, we used fibroblasts from a patient with a deficiency at the level of CPT1A. This patient carried a nonsense mutation in the *CPT1A* gene that leads to nonsense mediated decay of the mRNA, as evidenced by low *CPT1A* expression levels (Fig. 2), and to the likely absence of CPT1A protein. This cellular model allowed us to address the question whether CPT1A is crucial for the translocation of acylcarnitines across the OMM.

Mitochondria isolated from the CPT1A-deficient cell line were used to evaluate palmitoylcarnitine oxidation based on the levels of acetylcarnitine produced. We observed that palmitoylcarnitine oxidation is unaffected in the CPT1A-deficient cell line, in contrast to palmitoyl-CoA oxidation (Fig. 3). Interestingly, in control cell lines, malonyl-CoA reduced the oxidation not only of palmitoyl-CoA but also of palmitoylcarnitine. The inhibition of palmitoylcarnitine oxidation was however much less potent than the inhibition of palmitoyl-CoA oxidation, consistent with the established role of malonyl-CoA as the physiological inhibitor of CPT1 activity. In CPT1A-deficient cells, palmitoylcarnitine oxidation was not affected by the addition of malonyl-CoA (Fig. 3). These results suggest that malonyl-CoA is able to inhibit the oxidation of palmitoylcarnitine but that this phenomenon is dependent of the presence of the CPT1 protein. Despite this differential effect of malonyl-CoA, acylcarnitines are able to enter the mitochondria for β-oxidation independently from the presence or activity of CPT1A, suggesting that this enzyme is not crucial for the translocation of long-chain acylcarnitines across the OMM.

To further validate these results, another strategy was undertaken using an intact cell model in which an endogenously formed carnitine ester would be imported into the mitochondria for further oxidation. Using pristanic acid as substrate, we could monitor the mitochondrial uptake of acylcarnitines in two distinct ways: by analysing the acylcarnitine profiles in the external cellular medium after incubation of the cells with this fatty acid and by measuring the oxidation rate using radiolabelled pristanic acid. We showed that DMN-carnitine, the intermediate resulting from the peroxisomal degradation of pristanic acid, is successfully imported into the mitochondria of CPT1A-deficient fibroblasts (Fig. 4). Pristanic acid β-oxidation measurements in intact human fibroblasts revealed that CPT1A deficiency does not affect the mitochondrial import and oxidation of propionylcarnitine as well (Fig. 5).

Therefore, the studies in intact cells using pristanic acid as substrate complement the experiments with isolated mitochondria, providing further evidence that CPT1A is not essential for the translocation of long-chain acylcarnitines across the OMM or for the

mitochondrial import of branched medium-chain and straight short-chain acylcarnitines.

Our findings raise various questions. Is there a pore actually being formed by the alleged oligomerization of CPT1A? And if so, one can wonder what would be the alternative function of the CPT1A oligomer when it does not play an essential role in the transport of acylcarnitine intermediates across the OMM.

Surprisingly, our results show that malonyl-CoA, besides being an inhibitor of the transferase activity of CPT1, also seems to interfere with the oxidation of long-chain acylcarnitines. The relevance of this effect is unclear as the oxidation of palmitoylcarnitine is unaffected in the CPT1A-deficient cell line, suggesting that the translocation of the long-chain acylcarnitine species does not solely depend upon CPT1A. Nevertheless, our finding that the oxidation of acylcarnitines is modulated by malonyl-CoA suggests a novel mode of action for this inhibitor. The underlying mechanism is probably the result of a conformational change of CPT1A induced by the inhibitor. This conformational change could interfere with the putative channelling of acylcarnitines. We cannot formally exclude a role of CPT1A in the translocation of acylcarnitines. However, our results strongly indicate that this pathway is not exclusive as these intermediates are still able to access the mitochondrial matrix in the absence of CPT1A. As such, some other mechanism must be promoting the transport of acylcarnitines across the OMM. Alternatively, it has been hypothesised that acylcarnitines would be formed in the IMS [8]. This would be in agreement with the model proposed by Lee and co-workers [12] (Fig. 1B). We may postulate that VDAC could also be the player in the transport of acylcarnitines not synthetized by CPT1A. Nevertheless, this is an unlikely possibility, at least for long-chain acylcarnitines, as the oxidation of palmitoylcarnitine was shown not to be affected by VDAC inhibition [16]. Definite answers require the resolution of the CPT1A 3D structure which has been hampered by the hydrophobic nature of this protein. If CPT1A is not involved in the mitochondrial uptake of acylcarnitines, it is also unlikely to have a role in their export across the OMM. Thus, both import and export of acylcarnitines through the OMM remain unresolved with the currently available data, pointing to the existence of other mechanism(s) to translocate these intermediates into the mitochondrial matrix and from the mitochondria to the cytosol. Taken together, the results presented in this paper suggest that CPT1A is not involved in the channelling of acylcarnitines, or at least it is not the exclusive route for the transfer of the cytosolic acylcarnitine intermediates into the mitochondrial intermembrane space for further import into mitochondria and complete oxidation for energy production.

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Chapter 7

Peroxisomes contribute to the acylcarnitine production when the carnitine shuttle is deficient

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Submitted

Peroxisomes contribute to the acylcarnitine production when the carnitine shuttle is deficient

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Abstract

Fatty acid β-oxidation may occur in both mitochondria and peroxisomes. While peroxisomes oxidize specific carboxylic acids such as very long-chain fatty acids, branched-chain fatty acids, bile acids, and fatty dicarboxylic acids, mitochondria oxidize long-, medium-, and short-chain fatty acids. Oxidation of long-chain substrates requires the carnitine shuttle for mitochondrial access but medium-chain fatty acid oxidation is generally considered carnitine-independent. Using control and carnitine palmitoyltransferase 2 (CPT2)- and carnitine/acylcarnitine translocase (CACT)-deficient human fibroblasts, we investigated the oxidation of lauric acid (C12:0). Measurement of the acylcarnitine profile in the extracellular medium revealed significantly elevated levels of extracellular C10- and C12-carnitine in CPT2- and CACT-deficient fibroblasts. The accumulation of C12-carnitine indicates that lauric acid also uses the carnitine shuttle to access mitochondria. Moreover, the accumulation of extracellular C10-carnitine in CPT2- and CACT-deficient cells suggests an extramitochondrial pathway for the oxidation of lauric acid. Indeed, in the absence of peroxisomes C10-carnitine is not produced, proving that this intermediate is a product of peroxisomal β-oxidation. In conclusion, when the carnitine shuttle is impaired lauric acid is partly oxidized in peroxisomes. This peroxisomal oxidation could be a compensatory mechanism to metabolize straight medium- and long-chain fatty acids, especially in cases of mitochondrial fatty acid β-oxidation deficiency or overload.

Keywords: Fatty acid β-oxidation; Peroxisomes; Mitochondria; Acylcarnitines; Carnitine shuttle; Medium-chain fatty acids

Introduction

Fatty acid β-oxidation is an important source of energy production in mammals and may occur in both mitochondria and peroxisomes. Although the mechanism of mitochondrial and peroxisomal β-oxidation is similar, they serve different functions in the cell, evidenced by the different clinical manifestations associated with inherited disorders in mitochondrial [1] or peroxisomal β-oxidation [2]. After transport to the cell by proteins such as the plasma membrane fatty acid binding protein (FABPpm), the fatty acid translocase (FAT/CD36) and members of the fatty acid transport protein family (FATP), fatty acids may be activated to the respective acyl-CoA esters by one of the acyl-CoA synthetases existing in the cell [3]. These enzymes have different substrate specificities and tissue expression and are present at the cytoplasmic side of the plasma and mitochondrial membranes, within the mitochondrial matrix, in peroxisomes and in the endoplasmic reticulum [4]. Peroxisomal and mitochondrial β-oxidation essentially differs in

Abbreviations: C12:0, lauric acid; CACT, carnitine/acylcarnitine translocase; CPT1, carnitine palmitoyltranferase 1; CPT2, carnitine palmitoyltransferase 2; CrAT, carnitine acetyltranferase; CrOT, carnitine octanoyltransferase; L-AC, L-aminocarnitine; LCFA, long-chain fatty acids; MCFA, medium-chain fatty acids; POCA, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate.

substrate specificities and transport of substrates and products of β-oxidation across the membrane. Short- and medium-chain fatty acids allegedly undergo oxidation only in mitochondria and the β-oxidation of long-chain fatty acids is also believed to occur predominantly in this compartment. Peroxisomes oxidize specific carboxylic acids such as very long-chain fatty acids, branched-chain fatty acids, bile acids, and fatty dicarboxylic acids (DCAs). The fatty acids designated to undergo peroxisomal β-oxidation likely enter peroxisomes as acyl-CoA esters and the candidates to perform this transport are the ATP-binding cassette (ABC) transporters, proteins that couple ATP hydrolysis to substrate transport [5]. Mammalian peroxisomes contain three different half ABC transporters, including the adrenoleukodystrophy protein (ALDP or ABCD1), the ALD-related protein (ALDRP or ABCD2) and the 70-kDa peroxisomal membrane protein (PMP70 or ABCD3) [5, 6]. Although the individual functions of the peroxisomal ABC transporters remain to be established, it has been suggested that PMP70 is involved in the transport of long-chain fatty acids (LCFAs) across the peroxisomal membrane due to an increase in the rate of palmitic acid β-oxidation in cells overexpressing this protein [7]. Once in the peroxisome, acyl-CoAs are initially oxidized until the carbon chain is shortened to C8- and C6-CoA. These medium-chain substrates are the end products of peroxisomal β-oxidation and after conversion to acylcarnitines by carnitine octanoyltransferase (CrOT) or peroxisomal carnitine acetyltransferase (CrAT) are transported to mitochondria for further oxidation to acetyl-CoA. It has been shown that peroxisomes can handle long-chain acyl-CoAs with decreasing affinity to medium-chain substrates [8], but the contribution of these organelles to the oxidation of straight medium- and long-chain fatty acids is not clear.

Mitochondrial oxidation of LCFAs (generally considered from C14 to C20) requires conjugation with carnitine. After cytosolic activation of the LCFAs to the respective acyl-CoA esters, they are able to cross the mitochondrial outer and inner membranes by the action of the carnitine shuttle. This system is composed by carnitine palmitoyltransferase 1 (CPT1), carnitine/acylcarnitine translocase (CACT) and carnitine palmitoyltransferase 2 (CPT2). CPT1 converts the long-chain acyl-CoAs into the respective acylcarnitines which are further translocated through the inner mitochondrial membrane via CACT. In the mitochondrial matrix CPT2 reconverts them to the respective acyl-CoA which can enter the β-oxidation pathway for degradation and energy production [1]. Contrary to LCFA, the oxidation of medium-chain fatty acids (MCFAs; usually considered from C6 to C12) is believed to be largely independent of the carnitine shuttle [9-13]. Indeed MCFAs are able to cross the inner and outer mitochondrial membranes by diffusion and are activated to CoA esters by medium chain acyl-CoA synthetases localized in the matrix [14]. Nevertheless, due to the broad specificity of the cytosolic long-chain acyl-CoA synthetases, MCFA esterification may also occur in the cytosol, as suggested in previous studies showing MCFA elongation [15, 16]. Esterification of MCFA in the cytosol would involve the carnitine shuttle in the transport of these intermediates into mitochondria for further oxidation. Some studies point to stimulation of MCFA oxidation by carnitine [17-19], as well as altered plasma and urinary concentrations of carnitine esters following medium-chain triglyceride ingestion [20-22]. This suggests that carnitine may influence MCFA metabolism.

Here we show that the carnitine shuttle contributes significantly to the oxidation of the MCFA lauric acid (C12:0), as evidenced by its impairment in CPT2- or CACT-deficient cells. In this situation, lauric acid is directed instead to the peroxisome where it undergoes additional cycles of β-oxidation. This suggests that peroxisomes may have a role in the oxidation of straight medium- and long-chain substrates, particularly when mitochondrial fatty acid β-oxidation is defective or overloaded.

Materials and Methods

Materials

Dulbecco's modified Eagle's medium (DMEM), minimal essential medium (MEM), penicillin, streptomycin and Hepes were obtained from Gibco Invitrogen (Carlsbad, California, USA). Fetal bovine serum and L-glutamine were from BioWhittaker Lonza (Verviers, Belgium). Lipofectamine 2000, Trizol and the Superscript II Reverse Transcriptase kit were purchased from Invitrogen (Carlsbad, CA, USA). The LC480 Sybr Green I Master mix was from Roche (Mannheim, Germany). The [U-^{13}C]-palmitic acid (C16:0) was obtained from Advance Research Chemicals (Catoosa, OK, USA) and D3-lauric acid (C12:0) was from Cambridge Isotope Lab (Andover, MA, USA). The internal standards D3-propionyl-(D3-

C3-), D3-octanoyl- (D3-C8-) and D3-palmitoyl-carnitine (D3-C16-carnitine) were synthesized by Herman ten Brink (VU Medical Center, Amsterdam, The Netherlands). For immunofluorescence analysis the biotinylated donkey anti–rabbit Ig was purchased from Amersham and the streptavidin-labeled fluorescein isothiocyanate (streptavidin-FITC) was obtained from Dako (Denmark).
Puromycin, human serum albumin (HSA), bovine serum albumin (BSA), ferrocenium hexafluorophosphate, L-cysteine, L-carnitine and acetylchloride were obtained from Sigma-Aldrich (St. Louis, MO, USA). All other chemicals were of analytical grade.

Cell culture conditions

Human skin fibroblasts were obtained from anonymized controls (n=4) and patients with established deficiencies at the level of CPT2 (n=1, p.[R124X];[R124X]), CACT (n=2, c.241G>A apparent homozygous on cDNA and c.[3G>A];[417+1G>T]), MCAD (n=2 p.[K329E]; [K329E] and p.[S245L]; [S245L]), PEX5 (n=1 p.[N526K]; [N526K]) and PEX19 (n=1 p.[M255NfsX24]; [M255NfsX24]).
HEK293 cells and primary skin fibroblasts were cultured in DMEM with 4.5 g/L glucose, 584 mg/L L-glutamine and 25 mM Hepes, supplemented with 10% FBS, 100 U/mL penicillin, 100 mg/mL streptomycin, 100 mg/mL fungizone, in a humidified atmosphere of 5% CO_2, at 37ºC.

Knockdown of peroxisomal biogenesis factor13

Peroxisomal biogenesis factor 13 (PEX13) shRNA containing viruses were produced in HEK293 cells using MISSION shRNA targeting the gene coding for PEX13 (Sigma-Aldrich TRCN0000083622). HEK293 cells were co-transfected with pMD2G, pMDL/RRE, pRSV/REV and the shRNA TRC vector targeting PEX13 using Lipofectamine 2000. After 24 h the medium was refreshed. In the next day the virus-containing medium was collected, centrifuged and stored at -80ºC prior to transduction in order to prevent contamination with HEK293 cells. Primary skin fibroblasts from controls and CPT2- and CACT-deficient cell lines were incubated with the virus-containing medium. After 24 h, the medium was refreshed and puromycin was added to a concentration of 5 µg/mL to select for transduced cells.

Immunofluorescence analysis of peroxisomes

Immunofluorescence analysis was performed essentially as described before [23]. Briefly, cultured fibroblasts were washed twice with 10 mg/mL BSA in phosphate buffered saline (PBS) and fixed for 20 min with 2% paraformaldehyde in PBS/0.1% Triton X-100. Cells were washed twice with PBS/0.1% Triton X-100, and free aldehyde groups were blocked by incubating for 10 min in 0.1 M NH_4Cl in PBS. Cells were washed three times with 10 mg/mL BSA in PBS and incubated for 45 min with anti-catalase [24]. Cells were washed three times with 10 mg/mL BSA in PBS, incubated for 30 min with biotinylated donkey anti–rabbit Ig and stained with streptavidin-FITC.

Incubation of human fibroblasts with fatty acids

The acylcarnitine profiling in human fibroblasts was performed essentially as described before by Ventura *et al* [25] with minor modifications. Fibroblasts were seeded in 48 well plates (approximately 12.5 µg protein per well) and incubated overnight at 37ºC. The following day, after washing the cells with PBS, the incubation mixture was added to each well and incubated in a humidified CO_2 incubator (5% CO_2, 95% air) at 37°C. Incubation mixtures contained MEM supplemented with BSA (essentially fatty acid free), 0.4 mM L-carnitine and 120 µM D3-lauric acid (C12:0) or [U-^{13}C]-palmitic acid (C16:0) to a final volume of 250 µL. For the inhibition studies, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate (POCA) and L-aminocarnitine (L-AC) were added to the incubation mixture to a final concentration between 0-10 µM and 0-2000 µM, respectively. After 72h, the medium was collected and the cells were washed and resuspended in 100 µL of PBS with 1 g/L Triton X-100 to measure protein content using the bicinchoninic acid assay [26] and HSA as standard.
To 50 µL medium, 560 µL acetonitrile was added containing 50 pmol of D3-C3-carnitine and 20 pmol D3-C16-carnitine internal standards. After derivatization of the produced acylcarnitines with 1-butanol/acetylchloride 4/1 (v/v), these intermediates were quantified by Electrospray Ionization Tandem Mass Spectrometry (ESI-MS/MS) essentially as previously described [27].

Results

Oxidation of lauric acid in human fibroblasts with a defect in the carnitine shuttle

Control and CPT2- and CACT-deficient human fibroblasts were incubated with lauric acid and the formation of acylcarnitines was measured in the culture medium. High levels of C10- and C12-carnitine were found in the medium of CPT2- and CACT-deficient cell lines, contrasting with control fibroblasts where these intermediates did not accumulate (Fig. 1A and 1B). Levels of octanoylcarnitine (C8-carnitine) were similar in all cell lines tested (Fig. 1C). The formation of laurylcarnitine (C12-carnitine) in CPT2- and CACT-deficient cells indicates that, despite being considered a MCFA, a substantial part of lauric acid is activated to C12-CoA and enters the mitochondria via the carnitine shuttle. The formation of decanoylcarnitine (C10-carnitine) was unexpected, because if lauric acid would enter the mitochondria without the involvement of the carnitine shuttle, as it is generally assumed, it should have been completely oxidized. Therefore our results suggest an extramitochondrial source for the production of C10-carnitine.

The acylcarnitine profiles in medium-chain acyl-CoA dehydrogenase (MCAD)-deficient fibroblasts after pharmacological inhibition of CPT1

To investigate the contribution of the carnitine shuttle to the oxidation of lauric acid, we used POCA, an irreversible inhibitor of CPT1 activity, which blocks the conversion of acyl-CoAs into acylcarnitines and therefore limits the access of fatty acids to the matrix via the carnitine shuttle. MCAD-deficient cells were incubated with lauric acid (C12:0) and palmitic acid (C16:0) in the presence of increasing concentrations of POCA, followed by the measurement of C8-carnitine accumulation in the medium. This enabled us to monitor the effect of increasing CPT1 inhibition on the oxidation of these substrates. As expected, higher concentrations of POCA completely inhibited the oxidation of C16:0, a LCFA which is not able to access the mitochondrial matrix by diffusion (Fig. 2). When the cells were incubated with C12:0, we found that full inhibition of CPT1 leads to approximately 75% decrease in the levels of C8-carnitine in the medium (Fig 2). This indicates that the majority of the C12:0 given to the cells actually relies on the carnitine shuttle to access the

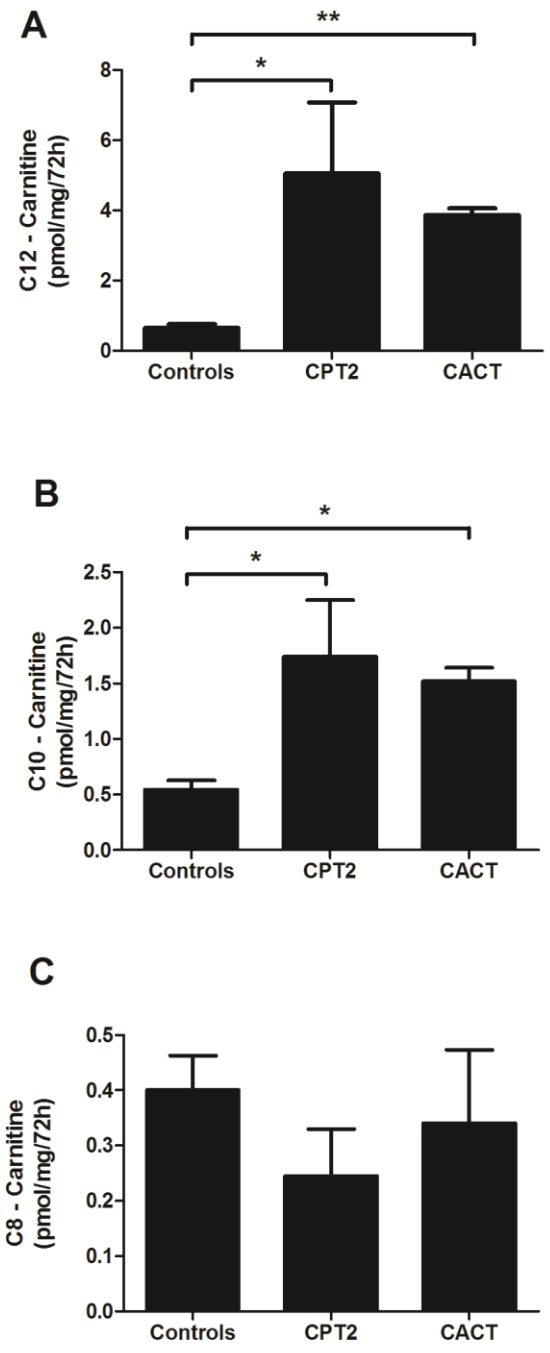

Fig. 1. Levels of the acylcarnitines present in the extracellular medium of human fibroblasts (control and CPT2- and CACT-deficient cell lines). A) C12-carnitine; B) C10-carnitine; C) C8-carnitine. Acylcarnitines were determined in the medium after incubation of the human fibroblast cell lines with lauric acid (C12:0) and L-carnitine. Data shown are mean ± SD of duplicates of at least two independent experiments. Experiments were performed with two independent cell lines in controls and CACT deficiency and in one cell line in the case of CPT2 deficiency. Significantly different values are marked with one or two asterisks (p value < 0.01 and p value < 0.001, respectively).

mitochondrial matrix and only 25% seems to be able to cross the mitochondrial membranes by diffusion.

The acylcarnitine profiles in control and CACT-deficient fibroblasts after PEX13 knockdown

We postulated that the C10-carnitine observed in the medium of CACT- and CPT2-deficient cell lines incubated with lauric acid could be the result of the involvement of peroxisomes in the oxidation of this MCFA. To investigate this hypothesis, we used control and CACT-deficient fibroblasts with and without knockdown of *PEX13*, a crucial gene in the peroxisome biogenesis [28]. The PEX13 knockdown efficiency was assessed by immunofluorescence using anti-catalase. Above 90% of the cells transduced with the PEX13 shRNA showed cytosolic catalase staining, confirming the absence of peroxisomes in these cells (results not shown). The fibroblasts transduced with the non-target shRNA showed a punctuated pattern that corresponds to the catalase within the peroxisomes (results not shown). Following this, all cell lines were incubated with lauric acid. In the CACT-deficient fibroblasts transduced with the non-target shRNA we observed a significant increase of C12-, C10- and C8-carnitine in the medium, compared to the normal controls (Fig. 3A, B and C). This accumulation is significantly reduced in the CACT-deficient cell line transduced with the PEX13 shRNA, with C10-carnitine showing the most pronounced decrease (Fig 3B), and C12- and C8-carnitine being reduced in parallel (Fig. 3A and 3C). The C6-carnitine intermediate was decreased as well, although not significantly (Fig. 3D). This illustrates that when the carnitine shuttle is deficient, lauric acid is partially metabolized in the peroxisome, leading to the production of medium-chain acylcarnitines with C10-carnitine being the most important end product.

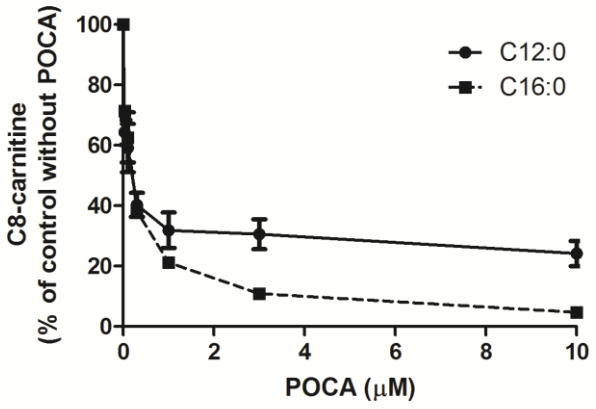

Fig. 2. Incubation of MCAD-deficient fibroblast cell lines with lauric acid (C12:0, solid line) and palmitic acid (C16:0, dashed line) and increasing concentrations of the CPT1 inhibitor POCA. The levels of octanoylcarnitine (C8-carnitine) present in the medium are represented as percentage of the control without POCA.

The acylcarnitine profiles in controls and Zellweger fibroblasts after pharmacological inhibition of CPT2

In order to confirm the results obtained for the oxidation of lauric acid in CACT- and CPT2-deficient fibroblasts, we used L-aminocarnitine (L-AC), a known inhibitor of CPT2 [29, 30]. Control fibroblast cell lines were incubated with lauric acid and increasing concentrations of L-AC, followed by the measurement of the acylcarnitine profile in the medium. Treatment with L-AC dose-dependently increased the levels of C12- and C10-carnitine (Fig. 4A and 4B, respectively), which is in line with the results obtained in the CACT- and CPT2-deficient cell lines. To provide additional evidence for the involvement of peroxisomes in the oxidation of lauric acid when the carnitine shuttle is compromised, we used fibroblasts from Zellweger patients which have a deficient peroxisome biogenesis due to a mutation in any of the PEX genes. In this case, we used cell lines with PEX5 and PEX19 deficiency. In the Zellweger cell lines incubated with lauric acid, the production of C10-carnitine is abolished upon CPT2 inhibition by L-AC (Fig 4B), proving that this intermediate is produced in the peroxisomes. C12-carnitine is lower after incubation with POCA, the irreversible inhibitor of CPT1 (Fig 4A). This confirms that the majority of the C12-carnitine observed in the medium of the fibroblasts after incubation with lauric acid, is the result of CPT1 activity.

Discussion

It is generally assumed that peroxisomes are not involved in the metabolism of straight-chain MCFAs. These substrates are normally oxidized in mitochondria and it is believed that their mitochondrial uptake is carnitine-independent [17-21]. Based on this assumption, CACT- and CPT2-deficient cell lines were incubated with lauric acid, a fatty acid generally considered a MCFA and which in theory would access the mitochondrial matrix by diffusion, followed by activation and further

oxidation. In the CACT and CPT2-deficient cell lines tested, high levels of C10- and C12-carnitine were observed in the medium, in contrast with the low levels found in control cells (Fig. 1A and 1B).

This extramitochondrial accumulation of C12-carnitine in CPT2 and CACT deficient cells suggests that lauric acid, at least *in vitro*, uses the the carnitine shuttle to access the matrix for further oxidation. In order to study the contribution of the carnitine shuttle to the oxidation of lauric acid, we incubated MCAD-deficient cells with lauric and palmitic acid and inhibited CPT1 with increasing concentrations of POCA. Incubation of an MCAD-deficient cell line with lauric or palmitic acid leads

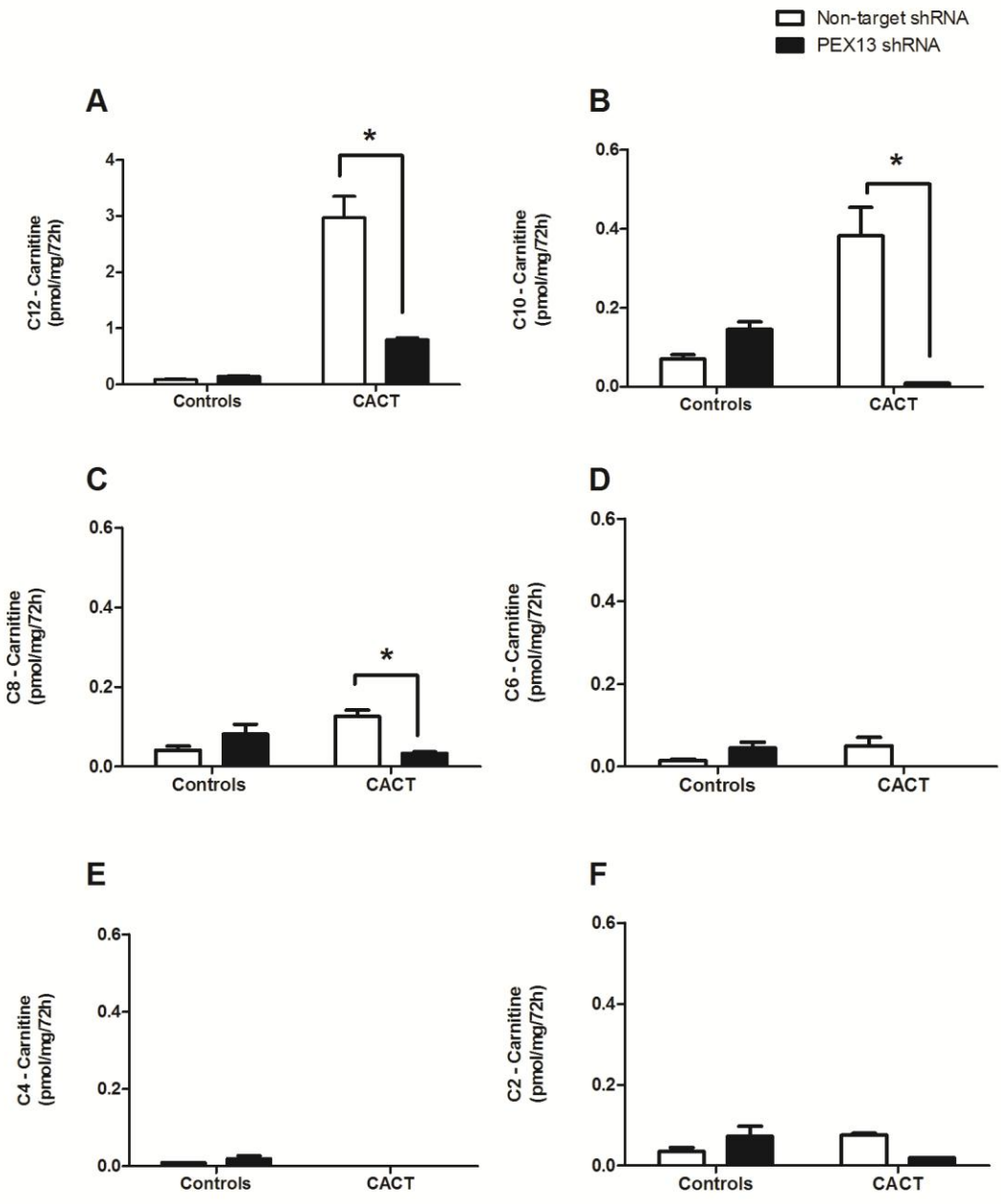

Fig. 3. Levels of the acylcarnitines present in the extracellular medium of human control and CACT-deficient fibroblasts with (solid bar) and without (open bar) PEX13 knockdown. A) C12-carnitine; B) C10-carnitine; C) C8-carnitine; D) C6-carnitine; E) C4-carnitine and F) C2-carnitine. Acylcarnitines were determined in the medium after incubation of the human fibroblast cell lines with lauric acid (C12:0) and L-carnitine. Data shown are mean ± SD of duplicates of two independent experiments. Experiments were carried with two independent control cell lines and one CACT-deficient cell line. Significantly different values are marked with one asterisk (*p* value ≤ 0.05).

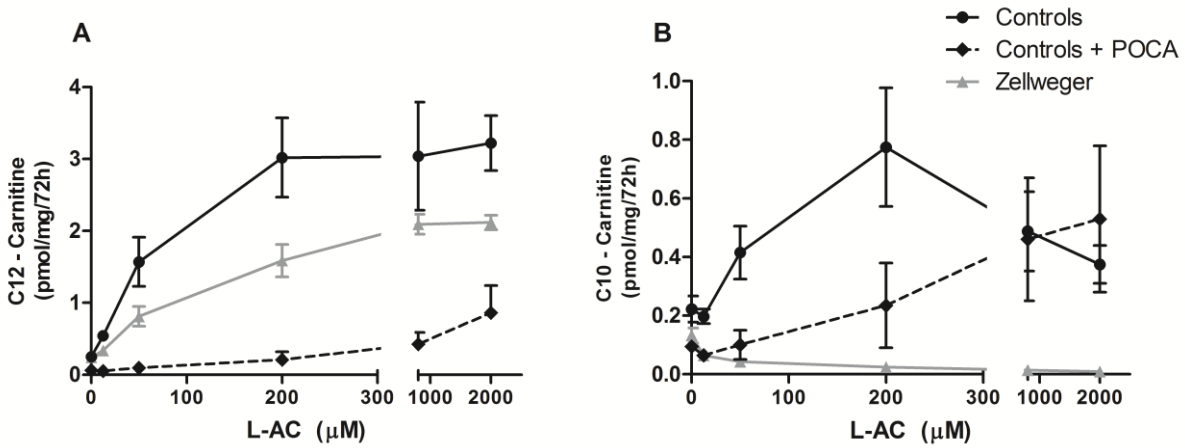

Fig. 4. Levels of the acylcarnitines present in the medium of human control fibroblasts (with and without 3 μM of the CPT1 inhibitor, POCA) and two fibroblast cell lines obtained from Zellweger patients (PEX5 and PEX19-deficient). A) C12-carnitine and B) C10-carnitine. Acylcarnitines were determined in the medium after incubation of the human fibroblast cell lines with lauric acid (C12:0) and L-carnitine. Data shown are mean ± SD of duplicates of at least two independent experiments. Experiments were carried out with two independent cell lines.

to the mitochondrial accumulation of C8-CoA followed by export to the medium in the form of C8-carnitine (Fig. 5). We observed that POCA inhibited the formation of C8-carnitine from lauric acid by approximately 75% (Fig 2), suggesting that CPT1 (and therefore the carnitine shuttle) strongly contributes to the oxidation of lauric acid. Nevertheless, a part of this substrate still enters the mitochondria by diffusion, even though this process does not seem to be the most relevant for its oxidation. When a shorter chain length substrate such as decanoic acid (C10:0) was used instead, the carnitine shuttle still contributed 50% to the mitochondrial oxidation of this fatty acid and again the diffusion process did not seemed to be the exclusive route for C10:0 entry into mitochondria [31].

If lauric acid would enter mitochondria by diffusion in CACT- and CPT2-deficient fibroblasts, its oxidation should be complete as there are no further mitochondrial defects in these cell lines. In this light, the extracellular accumulation of C10-carnitine observed in CACT- and CPT2-deficient cells incubated with C12:0 (Fig 1B) suggests an alternative extramitochondrial pathway for the oxidation of this fatty acid. In addition, we have recently demonstrated that the export of acylcarnitines from mitochondria is also dependent on the carnitine shuttle [31] which therefore also argues against mitochondria as the source of C10-carnitine in cells where the carnitine shuttle is compromised. Peroxisomes have been previously implicated in the oxidation of LCFAs and MCFAs, with activity decreasing from long- to medium-chain substrates and with C6-CoA as the presumed end-product of peroxisomal β-oxidation [8]. In order to explore the contribution of peroxisomes to the oxidation of lauric acid, we used fibroblasts from a CACT-deficient patient with normal and impaired peroxisomal biogenesis caused by *PEX13* gene silencing using a shRNA approach. Incubation with lauric acid in the CACT-deficient cells transduced with the non-target shRNA lead to the extracellular accumulation of C12-, C10- and C8-carnitine. Nevertheless, in the CACT-deficient cells with the *PEX13* knockdown, the extracellular levels of these acylcarnitine derivatives dramatically decreased, in particular for C10-carnitine (Fig. 3B). This strongly supports the involvement of peroxisomes in the formation of these medium-chain acylcarnitine species.

To further confirm these results, we used Zellweger fibroblasts from patients with deficiencies in crucial proteins for peroxisome biogenesis (PEX5 and PEX19). These cells were incubated with lauric acid and increasing concentrations of L-AC, an inhibitor of CPT2. Upon CPT2 inhibition, control cells showed elevated levels of C10-carnitine in the external medium (Fig 4B), validating the results obtained with the CPT2- and CACT-deficient cell lines. In the Zellweger fibroblasts the C10-carnitine intermediate is absent (Fig 4B), which confirms that the

peroxisomes are indeed the organelle responsible for the production of this acylcarnitine derivative. Thus, in cells with impairment at the level of CPT2 or CACT, lauric acid is (at least *in vitro*) directed to the peroxisome for further oxidation. *In vivo*, however, fatty acids also undergo ω-oxidation and in this case peroxisomes will handle the accumulating fatty acids mainly as dicarboxylic acids [32, 33].

The way by which the MCFA has access to the peroxisome is not clear but several routes may be postulated: (1) by diffusion; (2) after cytosolic activation to CoA ester and via ALDP, ALDR or PMP70; and/or (3) after conversion to acylcarnitine by CPT1 and reconversion to acyl-CoA inside the peroxisome by CrOT (Fig. 5). We show that when CPT2 is inhibited by L-AC in control cell lines, the levels of C10-carnitine can be decreased by the addition of POCA (Fig 4B). This suggests that lauric acid has better access to the peroxisome as C12-carnitine than as the acyl-CoA derivative. In the presence of POCA and at high concentrations of L-AC, the levels of C10- and C12-carnitine rise again. Since L-AC is only specific for CPT2 at low concentrations, we speculate that this effect may be related to the inhibition of other acyltransferases or transporters.

Once inside the peroxisome, C12-CoA will undergo one or two cycles of β-oxidation yielding C10- or C8-CoA. Further oxidation to C6-CoA in the peroxisome may still occur but at a much lower rate (Fig. 3D), probably due to the low specificity of peroxisomal β-oxidation to short-chain substrates [8]. Consequently, after this point, oxidation shifts to mitochondria for complete degradation of the short-chain intermediates via mitochondrial β-oxidation.

In the same model of pharmacological inhibition of CPT2, the accumulation of C12-carnitine in control cells incubated with lauric acid (Fig. 4A) supports

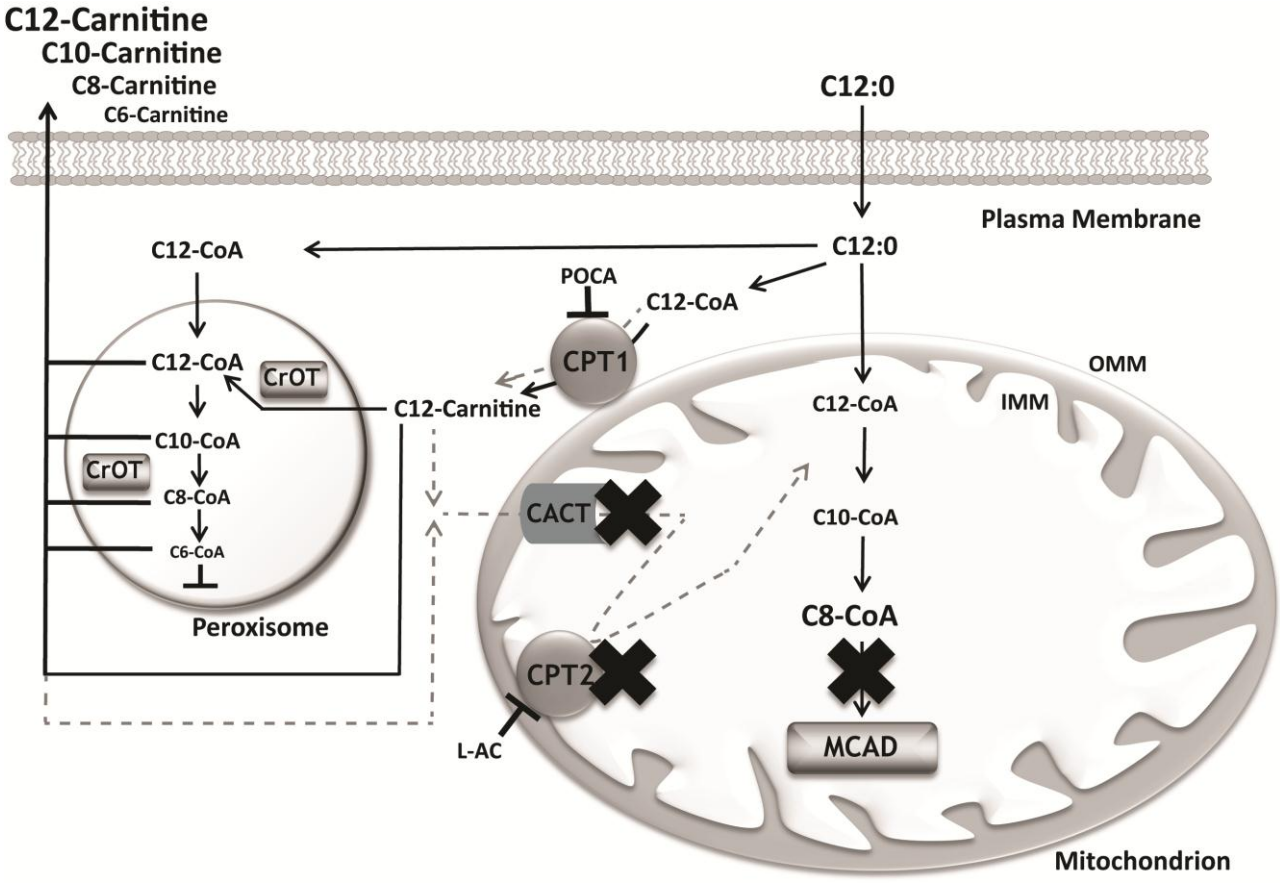

Fig. 5. Schematic representation of the proposed catabolism of medium-chain fatty acids (lauric acid, C12:0) by the peroxisomes in the case of carnitine shuttle impairment (CPT2 and CACT-deficient cells). Dashed arrows indicate the impaired flux when the carnitine shuttle is deficient. IMM, inner mitochondrial membrane; OMM, outer mitochondrial membrane; MCAD, medium-chain acyl-CoA dehydrogenase; CrOT, carnitine octanoyltranferase; L-AC, L-aminocarnitine; POCA, 2-[5-(4-chlorophenyl)pentyl]oxirane-2-carboxylate.

the cytosolic activation of lauric acid to lauroyl-CoA and its conversion to C12-carnitine by CPT1. In fact, after incubation with lauric acid, the levels of C12-carnitine decreased in Zellweger cells, but this intermediate is only virtually absent after incubation of the controls with the CPT1 inhibitor POCA (Fig. 4A). This confirms that the C12-carnitine observed is mostly originated from the activity of CPT1. In the case of carnitine shuttle impairment, C12-carnitine is either not able to access the mitochondrial matrix via CACT or it is not converted to C12-CoA because CPT2 is deficient and the affinity of the mitochondrial CrAT is low for substrates of 10 or more carbon atoms [34] being exported back to the cytosol. The activity of mitochondrial CrAT could also offer an explanation for the limited accumulation of C8- (Fig. 1C) and C6-carnitine (results not shown) in a CPT2-deficient cell line. These intermediates derived from the peroxisome could access the mitochondrial matrix followed by conversion to the respective CoA ester by CrAT and further complete oxidation by the mitochondrial β-oxidation. But this model is not consistent with the fact that C8-carnitine does not accumulate in the CACT-deficient cell line (Fig. 1C). Therefore we speculate that under the conditions tested, the main end-product of peroxisomal β-oxidation is C10-CoA.

In summary, our results confirm that lauric acid, as an example of a MCFA, can be esterified in the cytosol and that it further uses the carnitine shuttle to access the mitochondrial matrix. We also show that when the carnitine shuttle is defective, lauric acid can be partially oxidized in peroxisomes. We speculate that this would be also true for long- and other medium-chain fatty acids that do not typically undergo peroxisomal oxidation. Our findings suggest that peroxisomes are more important in straight MCFA oxidation than previously realized. Peroxisomal oxidation might serve as a compensatory mechanism in cases of mitochondrial fatty acid β-oxidation defects or mitochondrial overload.

Acknowledgments

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Chapter 8

A common X-linked inborn error of carnitine biosynthesis may be a risk factor for nondysmorphic autism

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A common X-linked inborn error of carnitine biosynthesis may be a risk factor for nondysmorphic autism

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We recently reported a deletion of exon 2 of the trimethyllysine hydroxylase epsilon (*TMLHE*) gene in a proband with autism. *TMLHE* maps to the X chromosome and encodes the first enzyme in carnitine biosynthesis, 6-*N*-trimethyllysine dioxygenase. Deletion of exon 2 of *TMLHE* causes enzyme deficiency, resulting in increased substrate concentration (6-*N*-trimethyllysine) and decreased product levels (3-hydroxy-6-*N*-trimethyllysine and γ-butyrobetaine) in plasma and urine. *TMLHE* deficiency is common in control males (24 in 8,787 or 1 in 366) and was not significantly increased in frequency in probands from simplex autism families (9 in 2,904 or 1 in 323). However, it was 2.82-fold more frequent in probands from male-male multiplex autism families compared with controls (7 in 909 or 1 in 130; *P* = 0.023). Additionally, six of seven autistic male siblings of probands in male-male multiplex families had the deletion, suggesting that *TMLHE* deficiency is a risk factor for autism (metaanalysis Z-score = 2.90 and *P* = 0.0037), although with low penetrance (2–4%). These data suggest that dysregulation of carnitine metabolism may be important in nondysmorphic autism; that abnormalities of carnitine intake, loss, transport, or synthesis may be important in a larger fraction of nondysmorphic autism cases; and that the carnitine pathway may provide a novel target for therapy or prevention of autism.

The role of carnitine in biology and disease has been studied for decades (1, 2). Carnitine has been proposed to be a conditionally essential nutrient, and even termed vitamin B_T. Carnitine content of foods varies widely, being very low in fruits, vegetables, and grains; intermediate in milk products, eggs, chicken, and fish; and very high in red meats. The proportion of carnitine derived from the diet varies widely in humans, being quite low in vegetarians and especially low with a vegan diet that excludes dairy products and eggs. In contrast, about 75% of carnitine is derived from the diet in meat eaters. Carnitine homeostasis in humans (Fig. 1) is maintained by a modest rate of endogenous synthesis, absorption from dietary sources, and efficient tubular reabsorption by the kidney. Apart from the dietary intake, carnitine is synthesized in humans in kidney, liver, and brain from protein-derived 6-*N*-trimethyllysine (TML) via 3-hydroxy-6-*N*-trimethyllysine (HTML), 4-*N*-trimethylaminobutyraldehyde (TMABA), and 4-*N*-trimethylaminobutyric acid [γ-butyrobetaine (γBB)] (3). Renal resorption plays an important role in carnitine metabolism, with considerable excretion if carnitine intake is abundant, but there is extremely efficient resorption if body stores of carnitine are low. Carnitine is present as free carnitine and as acylcarnitines, of which the latter reflect the cellular acyl-CoA ester pool. Up to 99% of carnitine is intracellular and is essential for mitochondrial function, where its role is to enable transport of fatty acids into mitochondria, where β-oxidation takes place (3); it is also involved in the transport of peroxisomal oxidation products to the mitochondria.

Carnitine deficiency can develop secondary to dietary inadequacy or as an adverse effect of medical treatments. Although humans can synthesize carnitine, nutritional deficiency can occur, as when infants were fed early preparations of soy formulas that were deficient in carnitine (4). Similarly, deficiency can arise with

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Conflict of interest statement: Multiple authors are based in the Department of Molecular and Human Genetics at Baylor College of Medicine, which offers extensive genetic laboratory testing, and Baylor College of Medicine derives revenue from this activity. The department offers biochemical and molecular diagnostic testing for trimethyllysine hydroxylase, epsilon gene deficiency. P.B.S.C.-S., S.V., F.M.V., and A.L.B. have filed a patent related to some of the work reported.

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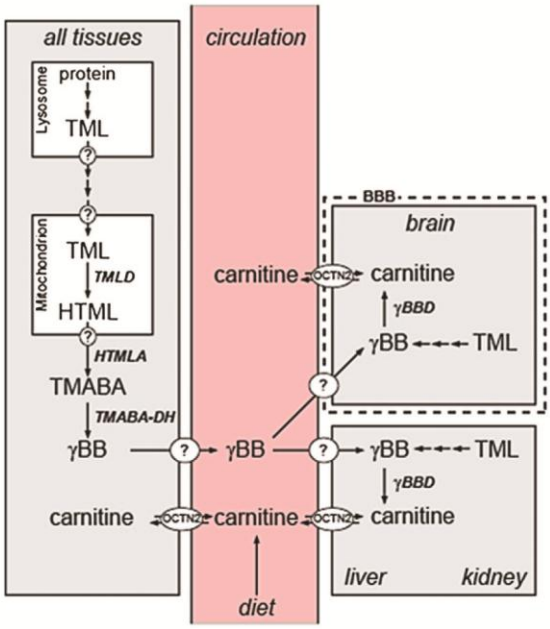

Fig. 1. Carnitine biosynthesis and homeostasis in humans. Carnitine is synthesized in four enzymatic steps. After release of TML by lysosomal protein degradation, this compound is hydroxylated by TMLD, producing HTML. HTML is cleaved by HTML aldolase (HTMLA) into TMABA and glycine. Subsequently, TMABA is oxidized by TMABA-dehydroxygenase (TMABA-DH) to form 4-*N*-trimethylaminobutyrate, also named γ-butyrobetaine (γBB). Finally, γBB is hydroxylated by γBBD, yielding L-carnitine. Because TMLD is located in mitochondria, TML needs to be transported out of the lysosome and across the inner mitochondrial membrane into the mitochondrial matrix by means of transporters, which are unknown at this time. Depending on the subcellular localization of the HTLMA (also uncertain, likely the cytosol), HTML or γBB needs to be transported back to the cytosol (where γBBD is located). In cells that do not contain γBBD, γBB is exported from the cell and imported into tissues (liver, kidney, and brain in humans) that do express γBBD by means of at least one specific transporter, presumably *SLC6A13*. Carnitine is transported by OCTN2 and other lower affinity transporters (not shown).

parenteral alimentation in neonates (5). Carnitine deficiency can also occur secondary to administration of pivalate-conjugated antibiotics or valproic acid (2). Various disease processes and medical interventions, such as renal tubular disorders and chronic hemodialysis, respectively, can also be associated with carnitine deficiency.

There are primary and secondary genetic forms of carnitine deficiency (6). Secondary deficiency is caused by various fatty oxidation defects and organic acidemias that lead to carnitine deficiency through urinary loss of acylcarnitines that accumulate related to the enzyme deficiency. Primary systemic carnitine deficiency is caused by biallelic loss-of-function mutations in the *SLC22A5* gene that encodes the plasma membrane organic cation transporter-2 (OCTN2). OCTN2 deficiency is characterized by excessive urinary loss of carnitine, leading to systemic deficiency with associated skeletal myopathy, cardiomyopathy, fatty liver, and hypoglycemia. Although the possibility of a primary systemic carnitine deficiency caused by a defect in carnitine biosynthesis was postulated long ago, no primary disorders of carnitine biosynthesis have been described until now (7).

Administration of carnitine is the centerpiece of therapy for systemic carnitine deficiency, and it is beneficial in some genetic forms of secondary carnitine deficiency. Administration of carnitine and acetylcarnitine has been explored as an antioxidant and for treatment of many disorders, including diabetic peripheral neuropathy (8), heart failure (9), and mitochondrial disorders.

Recently, we discovered a deletion of the 6-*N*-trimethyllysine dioxygenase (TMLD) gene [also known as trimethyllysine hydroxylase, epsilon (*TMLHE*)] while studying probands with autism, raising the question of whether there might be an association of autism with *TMLHE* mutations (10). *TMLHE* maps to the long arm of the X chromosome near the boundary of the pseudoautosomal region and encodes TMLD. TMLD is the first enzyme of the carnitine biosynthesis pathway (3) and is localized in mitochondria (11).

The etiology of severe, dysmorphic autism with a male/female ratio of 3.2:1 (12) has become increasingly well defined as often attributable to de novo mutations or recent mutations transmitted for a few generations. These mutations include large copy number variants (CNVs), which are detectable by chromosomal microarray analysis in up to 25% of the most severe cases with phenotypes that include major intellectual disability, which restricts reproduction (13). De novo point mutations are also being discovered as causes of autism using next-generation sequencing of genomic DNA (14). Disease-causing CNVs are found in ~10% of patients with intermediate phenotypes, often with less severe intellectual disability (15, 16). In these cases, penetrance may be incomplete and the phenotype can be highly variable, with diagnoses of intellectual disability, autism, schizophrenia, and idiopathic epilepsy seen with the same CNV (17–19). These examples typify single-locus conditions, perhaps with genetic and nongenetic modifier effects. Autism spectrum disorders and related neurocognitive phenotypes blend into even more complex genotype-phenotype relationships with evidence for two-hit or two-locus pathogenesis (20). At the milder end of the autism spectrum are patients who often have speech, have an intelligence quotient (IQ) ranging from low to the normal range, and are nondysmorphic. This milder population, some of whom meet diagnostic criteria for Asperger syndrome, can display up to an 8:1 male/female ratio (21, 22), and will be referred to herein as having nondysmorphic autism (NDA). This includes the milder portion of the autism spectrum, but patients who have NDA can have severe cognitive and behavioral phenotypes. The etiology of NDA remains almost completely unknown, but the extreme sex ratio may provide a clue as to its etiology.

In this paper, we show that *TMLHE* deficiency is a very common inborn error of metabolism in males and suggest that it may be significantly more frequent in autistic male-male sib pairs than in controls.

Results

Deletions of Exon 2 Are Heterogeneous and Common in Autistic and Healthy Males. Given the discovery of a deletion of exon 2 in *TMLHE* in a male simplex proband with autism (10), we examined the frequency of *TMLHE* mutations in autism and control populations. We studied simplex families primarily from the Simons Simplex Collection (SSC) and multiplex families primarily from the Autism Genetic Resource Exchange (AGRE) collection, and we recruited multiple collaborators to study additional families (Table 1). We have now identified a total of 16 male autism probands, six affected male siblings of probands, and 24 healthy adult males with deletions of exon 2, indicating that this is a relatively common CNV (Table 1). For 28 of the 29 deletions characterized more thoroughly, size ranged from 5.7 to 15.9 kb and only exon 2 was deleted; one additional deletion of 59.6 kb removed exons 2–6 (Fig. 2 *A* and *B* and Table S1). Based on position, size, and sequence, there was a minimum of 14 different deletion junctions among 29 unrelated families. Sequencing of the breakpoints of many deletions showed that almost all junctions occurred in long interspersed elements and short interspersed elements in the introns flanking exon 2 (Fig. 2*B* and Table S2), as has been seen in other loci (23). For all SSC samples (probands, heterozygous mothers, and healthy fathers), the deletions were present in both DNA extracted directly from blood and DNA extracted from lymphoblastoid cell lines (LCLs).

Table 1. Sources of male autism and control samples and methods of testing

	No.	Deletion
Simplex autism		
SSC	1,887	6*
SCAP	80	0
Houston	24	0
Toronto	328	3
Paris	333	0
New York	252	0
Totals	2,904	9
Male probands from male-male sibling pairs†		
AGRE + NIMH	752	7
Toronto	93	0
Paris	53	0
New York	11	0
Totals	909	7
Male probands from male-female sibling pairs†		
Paris	38	0
New York	5	0
Controls		
SSC, NIMH, and AGRE autism fathers	2,197	7
NIMH controls	897	3
BPR	49	1
Houston fathers	36	0
Multiplex fathers	615	0
WTCCC	3,018	9
Toronto	1,975	4
Totals	8,787	24

SCAP, South Carolina Autism Project; WTCCC, Wellcome Trust Case–Control Consortium.
*Screening for the deletion and confirmation were performed as described in *Materials and Methods*.
†Excludes affected sibling.

In addition, we identified an extremely common intronic deletion (Fig. 2*A*) that appeared on the basis of comparative genomic hybridization (CGH) array to be indistinguishable in all cases. This is equivalent to Database of Genomic Variants numbers 115349 and 104572, which appear to be identical, and/or to number 97130, which is very similar. The intronic deletion was present in 74% of 93 autism male probands and 71% of 48 control males examined, and it should not be misinterpreted as causing enzyme deficiency. This intronic deletion was present on 24 of 29 chromosomes from unrelated families with exon 2 deletion of *TMLHE* (Table S1).

Genomic sequencing of exons for *TMLHE* is complicated by the presence of two pseudoexons (7aP and 8aP) that are highly homologous to exons 7a and 8a, and are imbedded in a large inverted repeat downstream of *TMLHE* (24) (Fig. 2*C*). In addition, there are two alternative exons 7b and 8b, which are located between the two inverted repeats and have sequences unrelated to 7a and 8a. Sequencing of exons 1–8 of *TMLHE* from genomic DNA in 536 SSC autism male probands, 98 AGRE probands, and 443 National Institute of Mental Health (NIMH) controls identified very few point mutations (Table S3). In addition to exon 2 deletion, sequencing in a multiplex AGRE family (AU 0177) identified an arginine-to-glutamine change in codon 241 (R241Q) in the mother and unaffected half-brother of the two autistic males (Fig. S1). More recently, we have been able to study plasma from 156 male SSC probands for carnitine biosynthesis metabolites. We identified one male (Table S3) with biochemical abnormalities similar to those described below, and sequencing identified an R70H mutation likely causing *TMLHE* deficiency.

Exon 2 Deletion Results in Loss of TMLD Activity and Absence of TMLD Protein. The functional effect of deletion of exon 2 of *TMLHE* was examined as TMLD enzyme activity based on its role in carnitine biosynthesis. Cultured LCLs from males with deletion of exon 2 had low or undetectable TMLD enzyme activity, and heterozygous mothers had reduced activity compared with healthy males (Fig. 3*A*). Results from family AU 0177 were complex, with the two affected brothers with deletion of exon 2 having very low or undetectable enzyme activity but the unaffected mother and half-brother showing low activity but higher than that of the affected brothers (Fig. 3 *A* and *B*). The mother is a compound heterozygote and transmitted the R241Q mutation to the unaffected half-brother of the siblings with autism. Cells with exon 2 deletion also lacked immunodetectable protein by Western blot analysis (Fig. 3*C*). Many of the control and autism cell lines in Fig. 3*D* have the intron 1 deletion, and many do not.

Analysis of RNA from LCLs using RT-PCR revealed low levels of skipping of exon 2 in most samples and a stable transcript with complete absence of exon 2 in cells from males with deletion of this exon (Fig. S2*A*). Thus, nonsense-mediated decay is not prominent for the exon 2 deletion transcript. This was confirmed using a quantitative RT-PCR assay, which showed normal levels of transcript for the exon 5/6 junction in all samples but complete absence of the exon 1/2 junction in deletion samples (Fig. S2*B*).

Diagnostic Metabolite Abnormalities in Plasma and Urine. The two affected brothers from AGRE family AU 0177 had a normal facial appearance in childhood and were otherwise nondysmorphic. They both had normal plasma free carnitine levels (33 and 34 μmol/L, normal = 22–65 μmol/L) at the recent ages of 15 and 17 y. In urine of the affected brothers, HTML and γBB were undetectable and the excretion of TML was threefold that of controls (Fig. 4*A*). Plasma from the two brothers and from 5 SSC probands showed a significant increase in TML, complete absence of HTML, and severely reduced levels of γBB, except for one case (Fig. 4*B*). The (HTML + γBB)/TML ratio was very low in patients compared with control plasma and may be an excellent index of TMLD activity (Fig. 4*C*). These data indicate that *TMLHE* deficiency represents a unique inborn error of carnitine biosynthesis. To search for other evidence of a common *TMLHE* deficiency and for other defects in carnitine biosynthesis, urine from 29 SSC probands who did not have any known *TMLHE* mutations was studied and did not reveal any abnormal carnitine metabolites.

Sex Ratio in NDA Is Not Caused by a Common Inherited Mutation in *TMLHE*. It was important to determine whether there was a common mutation or epigenetic mechanism causing TMLD enzyme deficiency, and perhaps explaining the male predominance in some forms of autism. To address the possibility of a common but difficult to detect inherited mutation, we analyzed SNP data from Illumina arrays on 411 AGRE families; this revealed no evidence of linkage for *TMLHE*, which is the most telomeric gene on Xq that is not on Y, or for *VAMP7*, which is nearby but across the pseudoautosomal boundary and present on both the X and Y chromosomes. For the *TMLHE* region, a maximum nonparametric linkage score of 1.25 and logarithm of odds (LOD) score of 0.34 were observed for markers located within and flanking the *TMLHE* gene. For the *VAMP7* gene region, a maximum nonparametric linkage score of 0.76 and LOD score of 0.15 were observed. Thus, there was no evidence for linkage at either locus. This is not surprising, given the extensive genetic heterogeneity in autism.

***TMLHE* Deficiency Likely Is a Risk Factor for Autism.** Because deletion of exon 2 was more common than any other mutations detectable by genomic sequencing, and because it was associated with loss of enzyme activity, it was expedient to analyze a large series of autism cases and controls for exon 2 deletion. A PCR assay

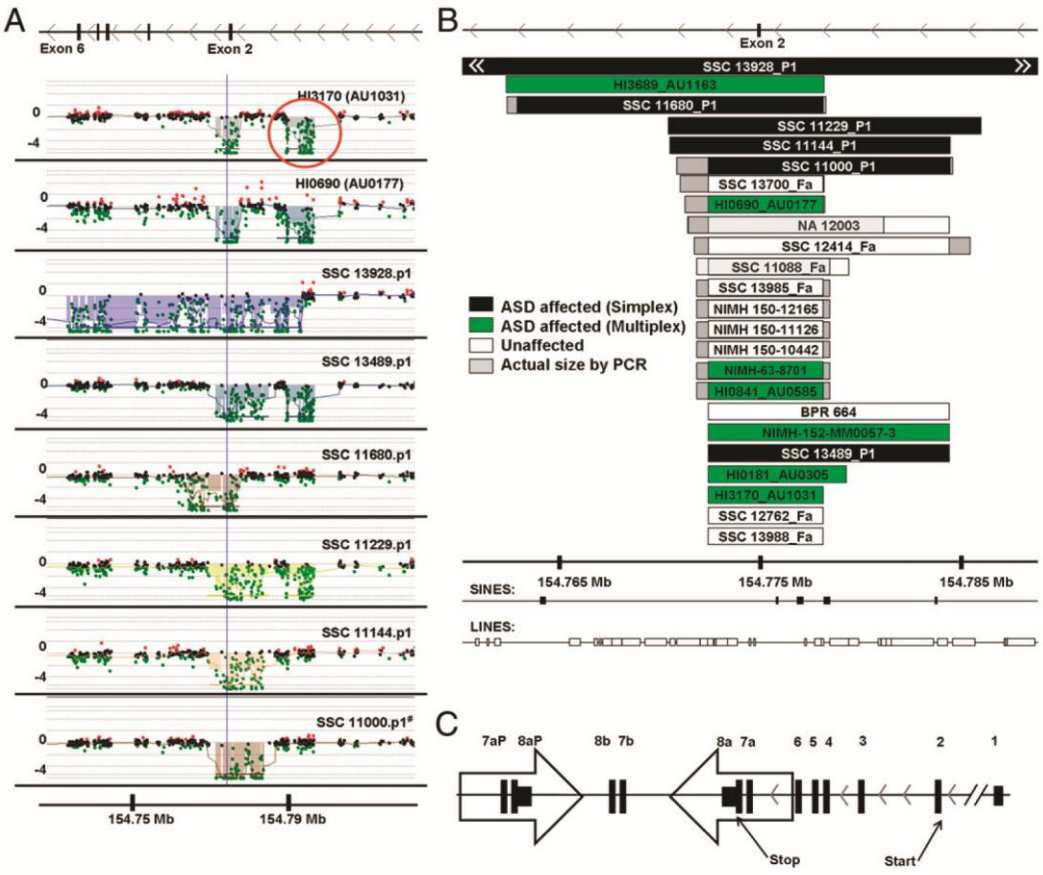

Fig. 2. Exon 2-containing deletions found in *TMLHE* and structure of *TMLHE*. (*A*) Array CGH showing eight exonic deletions in *TMLHE*, with the relative location of *TMLHE* exons 2–6 aligned above the array CGH plots (GRCh37/hg19 assembly; http://genome.ucsc.edu). The horizontal axis shows chromosome position, and the vertical axis shows the $\log_2$ ratio of array signal. Semitransparent filled boxes on CGH plots highlight the regions of aberration; all samples have deletions involving exon 2, and most have a separate deletion in intron 1 (red circle). All samples are autism probands with identifiers found in Tables S1, S4, and S5. (*B*) Twenty-four unrelated individuals with deletions involving exon 2 of *TMLHE* are mapped. Deletion coordinates were determined by array CGH unless specified by PCR assay. White arrowheads indicate the continuation of the deletion for SSC 13928.p1. NA 12003 is an unaffected individual whose deletion is published (38) and was better characterized in this study. BPR 664 is an unaffected individual. (*C*) Diagram of gene structure. Large open arrows represent near-identical inverted repeats. Fa, father; P1, proband; HI, AGRE individuals; #, individual first described by Celestino-Soper et al. (10).

was designed with primers slightly outside the boundaries of exon 2 to give a product of 538 bp in normal males but no product for males with deletion of exon 2. An example of the PCR assay with an internal control product is shown in Fig. S3. If a sample failed to give a PCR product for exon 2, the presence or absence of the deletion was then confirmed using CGH custom array with densely spaced oligonucleotides interrogating the *TMLHE* region, as shown in Fig. 2*A*. For PCR analysis, we focused entirely on males because the assay did not reliably detect the deletion in heterozygous females.

Using the PCR assay, we tested simplex male probands primarily from the SSC with lesser numbers of probands from the South Carolina Autism Project and from probands from Houston, TX. We also tested male controls, including SSC fathers of autism probands, NIMH controls, and Baylor Polymorphism Resource (BPR) controls. With the collaboration of the laboratories of two of the authors (J.S.S. and D.H.G.), we tested multiplex probands (here, multiplex refers to male-male sibling pairs both affected with autism) from the AGRE, NIMH, and Nashville collections. We subsequently developed collaborations with the laboratories of four of the authors (S.W.S., C.B., J.D.B., and M.E.H.) to expand the data for exon 2 deletion in autism male probands and control males. Some collaborating laboratories used quantitative PCR (qPCR) or existing Affymetrix 6.0 array data as the primary test for deletion of exon 2, as specified in Table 1. All deletion probands were validated, and approximate coordinates were determined using the custom *TMLHE* array. Deletions in control males from the laboratories of S.W.S. and M.E.H. were not validated.

Comparison of the data for male probands from simplex families (9 in 2,904 or 1 in 323 deleted) with all controls (24 in 8,787 or 1 in 366 deleted) did not provide evidence for an association ($P = 0.44$) (Table 1). One SSC proband (11680.p1) also had deletion of chromosome 16p11.2 and was eliminated from these calculations and from the phenotypic data. We hypothesized for numerous reasons reviewed in the discussion that the frequency of exon 2 deletion might be substantially higher in probands from male-male affected sib-pairs. The frequency of exon 2 deletion was 2.85-fold higher in these multiplex probands (7 in 909 or 1 in 130) compared with all male controls, with a P value of 0.023 (Table 1). For each multiplex family, only one affected male (identified as the proband) was tested initially. All genotypes were consistent with the X-linked inheritance; all 17 mothers of probands with deletion of exon 2 were tested and were heterozygous for the deletion, confirming that the deletions were not cell culture artifacts.

We next examined the affected male siblings of the multiplex male probands and found that six of the seven had the same deletion as the proband. Based on analysis using the Transmission Disequilibrium Test (TDT), the probability of obtaining this result, if there were no association, is 0.012. Using metaanalysis, we calculated a Stouffer's z-statistic to combine the data for multiplex probands compared with control males and data from the TDT. We obtained a Z-score of 2.90 and a P value of 0.0037 using Stouffer's method, suggesting that *TMLHE* deficiency is a risk factor for autism. If the data from the simplex families are included in the metaanalysis, the Z-score is −2.81 and the P value is 0.0051, which is only slightly higher than the P value of 0.0037.

Cognitive Function of *TMLHE*-Deficient Males with Autism Varies Widely. Significant phenotypic information was available for seven SSC probands, two of three Canadian Genetic probands, seven SSC unaffected fathers, all seven multiplex probands and six affected brothers, and 3 NIMH control males with deletion of exon 2 of *TMLHE* (Tables S4 and S5). The levels of cognitive and language functioning varied considerably across patients. The full-scale IQ of autistic males with deletion of exon 2 ranged from

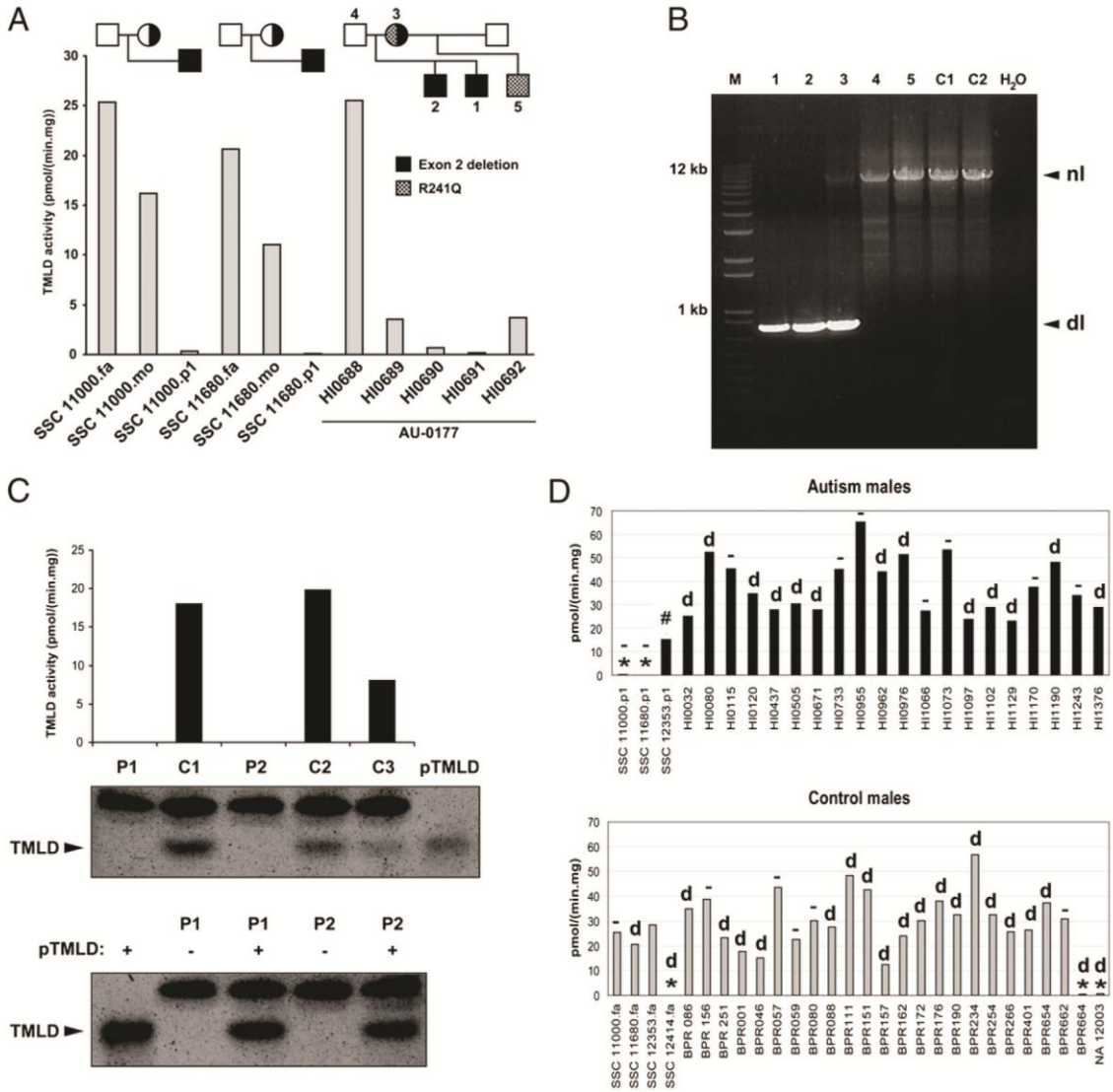

Fig. 3. Genetic and enzymatic characterization of hemizygous deletion of exon 2. (*A*) TMLD activity measured in lymphoblast homogenates of three families with exon 2 deletion. (*B*) PCR assay results for the AU 0177 family showing the deletion in the two affected brothers (1, 2) and in the mother (3) but not in the father (4), unaffected maternal half-brother (5), or unaffected controls (C1 and C2). There is bias of amplification in the mother, such that the normal band is faint. dl, deletion; nl, normal. (*C*) (*Upper*) TMLD activity and Western blot analysis of 2 individuals with exon 2 deletion (P1, HI0690; P2, BPR664) and three controls (C1–C3). Purified TMLD (pTMLD) is used as a positive control. (*Lower*) Western blot analysis of 2 individuals (P1 and P2) with (+) or without (–) addition of pTMLD, showing the complete absence of protein in cases of exon 2 deletion and confirmation of the identity of the immunoreactive material as TMLD. The upper band in the Western blot is an irrelevant protein. (*D*) (*Upper*) TMLD activity measured in lymphoblast homogenates from several autism males. *,*TMLHE* exon 2 deletion; #, E287K; d, deletion in intron 1 in 13 individuals; –, no deletion in intron 1 in 9 individuals. SSC 12353.p1 was not tested for the presence of intron 1 deletion. (*Lower*) TMLD activity measured in lymphoblast homogenates from male controls. BPR indicates local unaffected controls, and NA 12003 is an unaffected individual. SSC 12353.fa was not tested for presence of intron 1 deletion. There was no apparent correlation of the level of enzyme activity with the presence or absence of the intronic deletion. For *A*, *C*, and *D*, assays were run in duplicate and the average is plotted without error bars. fa, father; mo, mother; p1, proband.

38 to 143; 5 of 21 with available data were in the range of intellectual disability, and 3 of 21 were reported as untestable. One proband had seizures. For six of six cases in which information was available, patients were described as nondysmorphic. With respect to the controls, two of the seven SSC fathers had at least one domain with an elevated broader autism phenotype score based on the self-report Broad Autism Phenotype Questionaire, but the Social Responsiveness Scale rating by significant other and Family History Interview-Interviewee Impression scores were not consistent with the broader autism phenotype.

Assuming a True Association, the Penetrance for Autism in *TMLHE* Deficiency Would Be Very Low. The majority of males with an exon 2 deletion in the US and UK populations are expected to be phenotypically "normal" as adults. *TMLHE* deficiency was present in slightly less than 1% of probands from male-male affected sibling pairs; thus, it would be present in substantially less than 1% of all cases of autism. If we assume an overall frequency of 1 in 100 for autism, with a 4:1 male/female ratio, a frequency of 1 in 350 for *TMLHE* deficiency in normal males, and a frequency of *TMLHE* deficiency of 1 in 250 or 1 in 150 in males with autism, the penetrance would calculate at 2.2% or 3.6%, respectively (*SI Materials and Methods* and Table S6).

Discussion

TMLHE deficiency is a previously undescribed inborn error of metabolism discovered about 100 y after Garrod described such conditions in his 1908 Croonian Lectures to the Royal College of

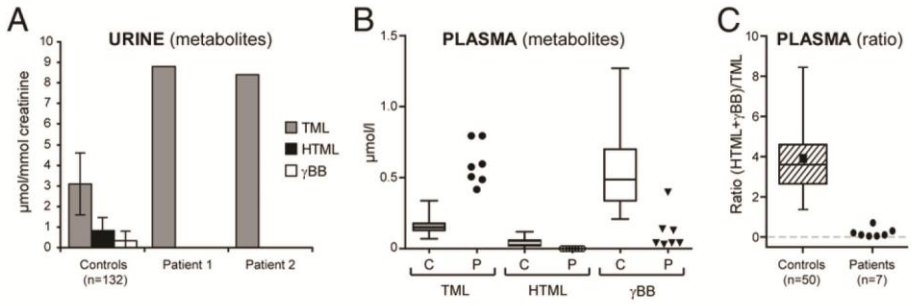

Fig. 4. Increased TML and decreased HTML and γBB in patients with *TMLHE* exon 2 deletions. (*A*) Bar diagram of concentrations (mean ± SD) of carnitine biosynthesis intermediates in urine of two patients with exon 2 deletion (1, HI0690; 2, HI0691) compared with controls. (*B*) Box and whisker plot of carnitine biosynthesis intermediates in plasma of seven patients with exon 2 deletion (HI0690, HI0691, SSC 13928.p1, SSC 13489.p1, SSC 11000.p1, SSC 11229.p1, and SSC 11680.p1) compared with controls. (*C*) Box and whisker plot showing the diagnostic potential of the (HTML + γBB)/HTML ratio as an indicator of *TMLHE* deficiency. All seven patients have a very low ratio. The black square represents the mean of the controls, and whiskers show the minimum and maximum values of the control group.

Surgeons. The frequency of *TMLHE* deficiency is startling, at ~1 in 350 control males of European descent, making it at least 20-fold more frequent than phenylketonuria in males. The enzyme deficiency and metabolite changes in plasma and urine are typical for an inborn error of metabolism. *TMLHE* appears to be a gene in which deletions are much more common than point mutations. There is precedent for this at the *DMD*, *PMP22*, *UBE3A*, and other loci causing Duchenne muscular dystrophy, hereditary neuropathy with liability to pressure palsies, Angelman syndrome, and other phenotypes, respectively. These biases are usually explained, in part, by genome architecture, as is likely the case for *TMLHE*.

It might be of some concern that we did not detect any indication that *TMLHE* deficiency is a risk factor for simplex autism. However, simplex and multiplex groups of autism families are significantly different, with an expectation of higher rates of de novo mutations in simplex compared with multiplex families and a higher rate of inherited mutations in multiplex vs. simplex families. In addition, shared genetic modifiers and shared environment are potential factors in multiplex families. Given these differences between simplex and multiplex families, the apparent low penetrance of *TMLHE* deficiency for autism, and the modest sample size, the negative result for simplex families is not surprising. Assuming that the association with multiplex autism is replicated, we would expect that there would be a significant association with simplex autism males with a much larger sample size, because all multiplex families are initially simplex before the birth of a second affected sibling. We would argue that it is not appropriate in this circumstance of low penetrance to combine the probabilities from simplex and multiplex families for an association of *TMLHE* deficiency with autism, because they are significantly different samples. However, if one were to do so, the failure to detect an association in a sample of simplex families of this size, given the necessarily low penetrance, has relatively weak statistical significance and does not detract substantially from the *P* value of 0.0037 observed with male-male multiplex families. We conclude that *TMLHE* deficiency is likely to be a weak risk factor for autism, but replication studies are needed, particularly those focusing on male-male multiplex families. The data make it clear that *TMLHE* deficiency is neither necessary nor sufficient to cause autism. With roughly 4 million births per year in the United States, this would equate to about 5,600 deficient males born per year, which, in turn, would equate to 168 males with *TMLHE* deficiency and autism assuming a 3% penetrance.

One might ask whether carnitine metabolism plays a broader role in the etiology of NDA. One possibility is that *TMLHE* deficiency is entirely benign, as is generally believed to be the case for pentosuria and histidinemia. Alternatively, *TMLHE* deficiency could mediate harmful effects either through toxic accumulation of TML or through deficiency of downstream metabolites, including HTML, TMABA, γBB, or carnitine. All these are possible, but we believe that the most attractive hypothesis at this time is that there is an increased risk for autism, and that this risk is modified by dietary intake of carnitine from birth through the first few years of life. Carnitine intake of the pregnant or nursing mother could also be important. There are extensive reports of mitochondrial abnormalities in autism, as reviewed recently (25), and some of the mitochondrial dysfunction could be secondary to carnitine deficiency. There are reports of low plasma carnitine in autism (26–29), but these reports have not prompted intensive investigations into a possible role of carnitine deficiency in autism and further studies are needed.

Another hypothesis could be that other genetic abnormalities involving the carnitine pathway might confer a risk for autism. Features of autism generally are not reported in children with systemic carnitine deficiency, although cases of autism with carnitine deficiency have been reported (26). Given the neurological basis of autism and the prominent expression of *TMLHE* in hippocampal neurons and Purkinje cells, one possibility would be that symptoms of autism might be secondary to carnitine deficiency in the brain. If that were the case, the pathophysiology of systemic carnitine deficiency would be very different from *TMLHE* deficiency. The former has low plasma carnitine, but ability to synthesize carnitine in the brain and elsewhere is intact. In the latter, plasma carnitine may be normal or low-normal based on dietary intake, but neurons are unable to synthesize carnitine and become completely dependent on transfer across the blood–brain barrier. If carnitine deficiency in the brain was deleterious, dietary deficiency, excess renal losses, disorders of transport (especially across the blood–brain barrier), and defects in synthesis might be risk factors for autism. Relatively little is known about transport across the blood–brain barrier, but this transport may be a limiting factor, because the concentration of carnitine in cerebrospinal fluid is 10- to 15-fold lower than in plasma (30, 31). As shown in Fig. 1, not all tissues are capable of complete carnitine biosynthesis because of the differential expression of the last enzyme, γBB dioxygenase (γBBD), which is only expressed in kidney, liver, and brain in humans. After degradation of proteins that contain TML residues, TML is converted to γBB, which is then transported to the tissues that express γBBD and converted into carnitine. The plasma membrane γBB transporter likely is encoded by the *SLC6A13* gene, which is known as a betaine/GABA transporter and has recently been suggested to function in carnitine biosynthesis as the liver γBB transporter (32). Transport of either or both carnitine and γBB across the blood–brain barrier could be important.

One important question is whether the association with autism is valid and can be replicated in future studies. Although

TMLHE deficiency was discovered by a genome-wide molecular analysis, a *P* value of genome-wide significance is not needed here, because this is a simple test of the hypothesis that a newly discovered inborn error of metabolism is a risk factor for autism or not. The metaanalysis *P* value of 0.0037, indicating an association with multiplex autism, suggests that the data indicating an association are unlikely to have occurred by chance. If penetrance for autism is influenced by carnitine intake during infancy, the risk for autism associated with *TMLHE* deficiency may be greater in countries with a high frequency of vegetarian diets and lower meat or beef intake. China, India, and South Korea are all countries where some studies of the incidence of autism are available (22, 33, 34) and there is a more vegetarian diet and/or much lower beef intake.

Two clinical investigations are of immediate interest and are being initiated. One is studying carnitine metabolites in cerebrospinal fluid of infants with autism with and without *TMLHE* deficiency near the age of onset, and the other is treating very young infants with autism with and without *TMLHE* deficiency with carnitine or γBB supplementation. Whether increased carnitine intake before onset of autism might prevent the development of symptoms would require a more complex study. There is a recent report of a trial of carnitine supplementation in autism suggesting clinical improvement (35), but the study included some patients up to 10 y of age who might be unlikely to respond; it would be desirable to have data from very young patients, preferably nondysmorphic, with and without *TMLHE* deficiency. The data reported here suggest that *TMLHE* deficiency is a risk factor for NDA and that carnitine metabolism could be a target for therapeutic intervention in this and related disorders.

Materials and Methods

Human Subjects and DNA. All work with direct involvement of human subjects was approved by the relevant institutional review boards or equivalents, and informed consent was obtained from all subjects. For SSC, AGRE, and NIMH samples, DNA derived from LCLs was obtained from the Rutgers University Cell and DNA Repository. Additional information is provided in *SI Materials and Methods*. The numbers of simplex probands, multiplex male-male sib pairs, and controls from various sources are specified in Table 1. Detailed information for cell culture and for identification of deleted probands and controls is given in *SI Materials and Methods*. For the screening of samples included in Table 1, we used PCR assays (Fig. S2), except for SSC samples, where Illumina arrays (36) were also used; for Toronto and the Wellcome Trust Case–Control Consortium, where Affymetrix 6.0 arrays were used; and for Paris and New York, where qPCR assays were used. All deletions in patients with autism were confirmed using custom arrays for the *TMLHE* region (Fig. 2*A*).

PCR and Sanger dideoxy-sequencing of *TMLHE* exons 1–8 was performed for 536 SSC male probands, 98 affected AGRE males from male-male multiplex families (brothers or half-brothers with the same mother), and 443 NIMH male controls (primers provided in Table S7).

CGH Array. All arrays used in this study were designed and analyzed based on University of California, Santa Cruz (UCSC) Genome Browser hg18 (National Center for Biotechnology Information Build 36, March 2006). The coordinates found in tables and figures are converted to hg19 (Genome Reference Consortium: human GRCh Build 37, February 2009). An Agilent CGH custom array of design ID 028249 was used to confirm *TMLHE* deletions originally found by PCR assay or those that were detected by the 1M Illumina SNP array through a collaborative study of SSC families (36). The custom array design is available on the Agilent's eArray website (www.agilent.com/genomics/earray). Analysis of CNVs was done using Agilent's DNA Analytics software (version 4.0.76) with the following settings: aberration algorithm ADM-2, a minimum of three consecutive probes per region, and a minimum absolute average $\log_2$ ratio of 0.25 for any given region.

The protocol for DNA digestion, labeling, purification, and hybridization to the arrays followed the manufacturer's instructions with some modifications, as described previously (37). Genomic DNA (800 ng) from the SSC individual and from a single male reference was used in the digestion. Each slide was scanned into an image file using the Agilent G2565 DNA Microarray Scanner at a 3-μm scan resolution. Each image file was quantified using Agilent Feature Extraction software (version 10.7.3.1). The Agilent custom-focused validation files were uploaded into the DNA Analytics software for analysis.

Enzyme Assays and Metabolite Determinations. All individuals tested for TMLD enzyme activity were assayed for the presence or absence of exon 2 of *TMLHE* by PCR or CGH array. These included BPR controls, AGRE and SSC individuals, and a Centre d'Étude du Polymorphisme Humain control (NA12003) (38). TML was obtained from Sigma–Aldrich. [$^2H_9$]TML and [$^2H_3$]γBB were synthesized as described previously (11). [$^2H_9$]HTML was prepared enzymatically by incubating [$^2H_9$]TML with *Neurospora crassa* TLMD, heterologously expressed in *Saccharomyces cerevisiae* as described previously (39). The resulting mixture of [$^2H_9$]HTML and [$^2H_9$]TML was applied to Amicon Ultra 30-kDa filters (Millipore), and the deproteinized filtrate was used as an internal standard for TML and HTML. All other reagents were of analytical grade.

Lymphoblast pellets were homogenized in 10 mM Mops buffer containing 0.9% (wt/vol) NaCl, 10% (wt/vol) glycerol, and 5 mM DTT (pH 7.4). The protein concentration was determined by the method of Bradford (40) using human serum albumin as a standard. For measurement of TMLD and γBBD activities, the reaction mixture consisted of 20 mM potassium phosphate buffer containing 50 mM KCl, 3 mM 2-oxoglutarate, 10 mM sodium ascorbate, 0.5 mM DTT, 0.5 mM ammonium iron sulfate, 2.5 mg/mL BSA, 2 mM TML, and 0.2 mM [$^2H_3$] γBB at pH 7.4, with a final volume of 250 μL. The reaction was started by adding 50 μL of homogenate (target final protein concentration of 0.2 mg/mL for lymphoblast homogenates) to the reaction mixture and was incubated at 37 °C for 30 min. The reaction was terminated by the addition of $ZnCl_2$ to a final concentration of 1 mM, and the reaction mixtures were placed on ice. The $ZnCl_2$ solution also contained the following internal standards: 50 pmol of [$^2H_9$]HTML, 140 pmol of [$^2H_9$]TML, 140 pmol of [$^2H_3$]γ-BB, and 550 pmol of [$^2H_3$]carnitine. Subsequently, the reaction mixture was loaded onto an Amicon Ultra 30-kDa filter and centrifuged at 14,000 × *g* for 20 min to separate the metabolites (TML, HTML, γ-BB, and carnitine) from the enzymes and remove most of the proteins. The filtrate (100 μL) was derivatized with methylchloroformate, and the produced HTML was quantified using ion-pair ultra performance liquid chromatography (UPLC)-tandem MS essentially as previously described (11).

For determination of carnitine biosynthesis metabolites in plasma and urine, internal standards were added to each homogenate and derivatization was performed as described above. Plasma samples were deproteinized using an Amicon Ultra 30-kDa filter. Urine samples were directly derivatized, and TML, HTML, carnitine, and γ-BB were quantified using ion-pair UPLC-tandem MS as previously described (11). For immunoblot analysis, a Multiphor II Nova Blot electrophoretic transfer unit (Amersham Pharmacia Biotech) was used to transfer proteins onto a Protran nitrocellulose membrane (Whatman) as described by the manufacturer. After blocking of nonspecific binding sites with 50 g/L Protifar (Nutricia) and 10 g/L BSA in PBS with Tween 20 (1 g/L) for 1 h, the membrane was incubated for 2 h in the same buffer without Protifar with 1:3,000 dilution of rabbit polyclonal antibodies raised against human recombinant TMLD fused to maltose-binding protein (41). Detection was performed with IRDye 800-conjugated goat anti-rabbit antibody (LI-COR Biosciences) according to the manufacturer's instructions. Membranes were then dried and scanned using the Odyssey Infrared Imaging System (LI-COR Biosciences).

TDT, Metaanalysis, and Penetrance Calculations. After 7 of 909 probands from multiplex male-male families were identified, the *P* value favoring a risk relationship of *TMLHE* deficiency to autism was 0.022 based on a one-sided Fisher's exact test. This outcome could have occurred by chance or could have occurred because there is indeed a risk relationship. If the result occurred by chance, 3.5 of the seven siblings would be expected statistically to have the deletion, if all mothers are assumed to be carriers. If the result reflects a true risk relationship, a higher proportion, but not necessarily all, of the autistic siblings should have the deletion. Seven of the eight siblings carried the deletion. Statistical analysis of the sibling data was performed by implementing the TDT (42–45). Because the X chromosome is being analyzed, only transmissions from the mother are informative. We examined whether or not the deletion had been transmitted to the affected male sibling from his mother and applied McNemar's χ^2 (46) to the resulting 2 × 2 table to guard against significant results attributable to population substructure/admixture. To combine the results from the comparison of multiplex probands to controls ($P = 0.023$) and from the TDT analysis of affected male siblings ($P = 0.012$), metaanalysis was performed using Stouffer's method (47). The metaanalysis resulted in a Z-score of 2.90 and a *P* value of 0.0037.

For estimating penetrance, we used a hypothetical population of 2 million individuals at risk with an equal number of males and females, as shown in Table S6. We assumed a frequency of autism of 1 in 100 with a 4:1 male/female ratio. We assumed that 1 in 350 normal males carried deletion of exon 2 of *TMLHE*. We then calculated the penetrance assuming that either 1 in 250 or 1 in 150 males with autism carries the deletion.

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Chapter 9

Concluding remarks and Perspectives

Concluding remarks

The importance of carnitine for energy metabolism and cellular homeostasis is firmly established, namely its pivotal role in the oxidation of long-chain fatty acids and in the formation of acylcarnitine derivatives. Nevertheless, several aspects of carnitine function and metabolism have not been addressed throughout the years, and many concepts have been generally assumed without formal proof. The main aims of this thesis were to elucidate the involvement of the proteins of the carnitine shuttle in the production and transport of the acylcarnitines generally present in the plasma of patients with mitochondrial fatty acid β-oxidation (mFAO) and branched-chain amino acid oxidation (BCAAO) disorders, as well as to understand the importance of the carnitine biosynthesis and homeostasis in neurological conditions such as autism spectrum disorders. The work here presented shows new data that provides answers to some of these questions.

The origin of acylcarnitines was one of our key research questions. It is commonly assumed that, following the accumulation of acyl-coenzyme A esters (acyl-CoAs) in mFAO and BCAAO defects, the enzymes carnitine palmitoyltransferase 2 (CPT2) and carnitine acetyltranferase (CrAT) are responsible for the mitochondrial conversion of these intermediates into acylcarnitines. However, the human enzymes had never been thoroughly investigated. Our extensive *in vitro* studies on the substrate specificity of these two acyltransferases showed that medium- and long-chain acylcarnitines are produced by CPT2 whereas the short-chain and the majority of the short branched-chain species are handled by CrAT, which nevertheless does not display any activity with dicarboxylic acyl-CoAs. We have therefore elucidated the origin of most of the acylcarnitines frequently found in the plasma of mFAO and BCAAO deficient patients. Interestingly, we further demonstrated that *trans-2*-enoyl-CoA esters are poor substrates for these acyltransferases as the double bond at the second position of the acyl moiety likely interferes with their catalytic mechanism. Furthermore, we have demonstrated that, at least for CPT2, *trans*-2-enoyl-CoAs may have an inhibitory effect upon the transferase activity. The potential deleterious effect of these *trans-2*-enoyl-CoAs upon CPT2 and CrAT may have important consequences in diseases where accumulation of these intermediates may occur. These disorders include mitochondrial trifunctional protein (MTP), isolated long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD), short-chain 3-hydroxyacyl-CoA dehydrogenase (SCHAD) or 2-methyl-3-hydroxybutyryl-CoA dehydrogenase (MHBD) deficiencies. These pathologies are generally more severe than other mFAO and BCAAO disorders and present unique features that distinguish them, namely neurological abnormalities and retinal damage [1, 2]. The causes for these severe features are yet unknown. The probable interference caused by the *trans-2*-enoyl-CoAs upon the acyltransferases' activity will prevent mitochondrial detoxification by inhibiting the formation of the respective acylcarnitines and promoting further acyl-CoA intramitochondrial accumulation. We speculate that the increased accumulation of acyl-CoAs within the mitochondria may be one of the causes for the severity of these diseases.

The crucial role of CPT2 in the synthesis of acylcarnitines, as well as the involvement of the carnitine/acylcarnitine translocase (CACT) and of the plasmmalemal carnitine transporter (OCTN2), respectively, in the mitochondrial and cellular export of these intermediates, was further addressed in our double knockdown cell lines. In this model the the gene encoding for the medium-chain acyl-CoA dehydrogenase was silenced in fibroblasts from CPT2- and CACT-deficient patients, inducing the mitochondrial accumulation of medium-chain acyl-CoAs. Although the reverse action of the carnitine shuttle has been hypothesized before and *in vitro* studies pointed in that direction, we have now clearly shown, in an intact cell model, the mitochondrial detoxification pathway after induced acyl-CoA accumulation. Unfortunately, the cellular export of these intermediates remains unknown. We hypothezised that OCTN2 could be the carrier responsible for this task. However, our data suggests that OCTN2 is not essential in the cellular export of acylcarnitines.

The transport of acylcarnitines across the outer mitochondrial membrane (OMM) remains also elusive. Previous studies predicted the oligomerization of carnitine palmitoyltranferase 1A (CPT1A) to form a pore-like structure, suggesting a

role for this enzyme in the channeling of acylcarnitines across the OMM [3, 4]. Our results indicate that even if CPT1A oligomerization leads to the formation of a pore in the OMM, this is not an exclusive pathway for the translocation of acylcarnitines to the intermembrane space since the absence of CPT1A protein does not affect the oxidation of acylcarnitines. Even though we should not exclude the participation of CPT1A in the channeling of acylcarnitines through the OMM, another mechanism must be involved as well. Surprisingly, our results show that malonyl-CoA, besides being an inhibitor of CPT1 activity, also interferes with the oxidation of long-chain acylcarnitines. The exact mechanism of CPT1A sensitivity to malonyl-CoA and consequent regulation of its activity is poorly understood but it has been suggested that an interaction of the inhibitor with the cytosolic N-terminal domain of CPT1 is crucial for this process [5, 6]. Furthermore, the modulation of malonyl-CoA sensitivity may be altered by interactions within the transmembrane domains which in turn affects the interaction between the N- and C-terminal domains [5, 7]. Similarly, interactions between the transmembrane domains have also been shown to be crucial for the formation of the CPT1A oligomeric structures and thus for the genesis of the hypothesized pore [4]. Binding of malonyl-CoA to CPT1A may induce conformational changes in the protein structure which would affect the transmembrane domains' interaction and thus the opening/closure mechanism of the putative pore, or even its formation by CPT1A oligomerization. Another hypothesis is that the synthesis of acylcarnitines by CPT1A takes place in the intermembrane space and not in the cytosol, thus obviating the need for a translocation mechanism for these metabolites. This has been suggested before [8, 9] but such model would require the passage of CPT1 substrates, i.e. large acyl-CoA units, through the OMM. The voltage dependent anion channel (VDAC) is the proposed candidate to mediate the transport of acyl-CoAs across the OMM since previous studies have shown that its inhibition affects the oxidation of palmitoyl-CoA but not of palmitoylcarnitine [10].

The role of peroxisomes in the production of acylcarnitines in cases of an impairment of the carnitine shuttle is also addressed in the work here reported. The oxidation of lauric acid, considered a medium-chain fatty acid (MCFA), is assumed to be carnitine-independent. MCFAs are believed to bypass the carnitine shuttle and enter the mitochondria by diffusion. Remarkably, our results demonstrate that the mitochondrial oxidation of lauric acid largely depends on the carnitine shuttle, since its oxidation is affected in CPT2 or CACT deficient fibroblasts. Moreover, when the carnitine shuttle is impaired, the peroxisomes are able to oxidize lauric acid until the formation of species with a shorter carbon chain (down to C8). These remarkable findings suggest that if the mitochondrial β-oxidation machinery fails, peroxisomes are able to handle the fatty acids that are typically oxidized in mitochondria, serving as a compensatory mechanism in cases of mFAO deficiencies.

Finally, we have identified the first inborn error of carnitine biosynthesis, *TMLHE* deficiency. This X-linked defect was found to be associated with non-dysmorphic autism and may be a risk factor for the development of this neurological disorder. Another recent study confirmed that *TMLHE* deficiency is associated with autism spectrum disorders (ASD) [11]. Biochemically, *TMLHE* deficiency is characterized by a compromised activity of the enzyme trimethyllysine dioxygenase (TMLD) and by increased levels of trimethyllysine (TML) and decreased levels of hydroxy-TML (HTML) and γ-butyrobetaine (γ-BB) in plasma and urine of patients. The structural resemblance of γ-BB (4-*N*-trimethylaminobutyric acid) to GABA (4-aminobutyric acid) and betaine (2-*N*-trimethylamino ethanoic acid) might point to a role of γ-BB other than in carnitine biosynthesis, possibly related to neurotransmission. In fact, dysfunction of GABAergic neurons has been suggested to contribute to numerous neuropsychiatric phenotypes, including autism [12]. Although the true impact of this carnitine biosynthesis deficiency and the underlying molecular mechanisms of the disease are not entirely clear, its association with ASD will encourage new lines of investigation which may provide novel therapeutic targets and preventive approaches for affected individuals.

Taken together our results reinforce the importance of carnitine and its metabolism in several processes

that are crucial for the cell, including the production and transport of acylcarnitines and its potential use as a therapeutic option for mild cases of autism. Nevertheless, many other questions remain unanswered and further studies are required to fully understand the mechanisms involved in the function and metabolism of carnitine.

Perspectives

As *trans*-2-enoyl-CoAs are poor substrates of acyltransferases, no acylcarnitine derivatives are likely formed in the mitochondria of cells were these intermediates accumulate due to an enzymatic block. The potentially deleterious action of these metabolites should be additionally investigated. The analysis of the acyl-CoA and acylcarnitine profile and other parameters such as cell viability should be further tested in MTP/LCHAD-deficient cell lines incubated with palmitic acid and L-carnitine. This may shed some light on the effect that the accumulation of these acyl-CoA or acylcarnitine intermediates might have upon the cell. Both LCHAD and VLCAD (very long-chain acyl-CoA dehydrogenase) deficiencies are accompanied by elevated lactate levels, suggesting a concomitant compromise of pyruvate metabolism in these pathologies. However, only LCHAD deficiency presents elevated lactate/pyruvate ratios. This differential effect upon pyruvate metabolism of the fatty acid β-oxidation intermediates specific of LCHAD and VLCAD deficiencies should be evaluated as well. It would be also interesting to understand the mechanisms underlying the neuropathies and retinopathies manifested in MTP/LCHAD deficiencies, but not in VLCAD deficiency, possibly by testing the effects of LCHAD knockdown in neuron or retinal cell lines.

The origin of the short-chain dicarboxylic acylcarnitines that appear in the plasma of patients with BCAAO deficiencies is still not resolved and should be further investigated since CrAT does not seem to be responsible for their formation. The use of cell lines from patients with deficiencies where these intermediates are expected to accumulate with and without CrAT or CrOT knockdown might be useful in such studies.

Although the mitochondrial synthesis of acylcarnitines and its export across the inner mitochondrial membrane is now elucidated, the import and export of acylcarnitines across the OMM remains elusive. The existence of another mechanism operating in the channeling of these carnitine derivatives, to and from the intermembrane space, should be thoroughly explored. Naturally, the investigation on the role of CPT1 in the transport of acylcarnitine intermediates across the OMM should be extended and pursued, possibly by using CPT1-deficient cell lines with an induced mitochondrial accumulation of fatty acyl derivatives. Studies on VDAC should also be performed to clarify if this protein is indeed involved in the channeling of acyl-CoA molecules across the OMM and, consequently, clarify if the formation of acylcarnitines is an event that takes place in the cytosol or within the intermembrane space. With this purpose, compounds that specifically inhibit VDAC could be tested in cell lines with mutations that lead to the absence of CPT1A protein or affect the formation of the putative pore, followed by evaluation of palmitoyl-CoA and palmitoylcarnitine oxidation. Furthermore, the potential effect of malonyl-CoA, revealed by our data, upon the oxidation of long-chain acylcarnitines is surprising and deserves further investigation. The use of cell lines with mutations that affect exclusively CPT1A activity or the creation by site-directed mutagenesis of mutations in specific residues that lead to subtle conformational changes within the regulatory domain of this enzyme should be used. This could help to better understand if the effect of malonyl-CoA on the oxidation of acylcarnitines is associated with alterations in the conformation of the protein. Definite answers to these questions likely require the resolution of the 3D structure of CPT1A and supplementary studies should also point in that direction.

Additionally, another question remains also unresolved: how are acylcarnitines exported from the cell? The plasma carnitine transporter, OCTN2, does not seem to be involved (or at least it is not the exclusive player) in this process. Further studies are required to unravel the transporter or mechanism behind the cellular export of acylcarnitines. Other transporters of the plasma

membrane should be investigated for this function, namely the ones responsible for the cellular uptake of free fatty acids such as the plasma membrane fatty acid binding protein (FABPpm), fatty acid transport protein (FATP) and fatty acid translocase (FAT/CD36).

We have reported for the first time the association of autism spectrum disorders (ASD) with *TMLHE* deficiency in male-male multiplex families but not in simplex families, which is consistent with the presence of a true, but weak, association. A substantially larger study should be performed to show an association with simplex autism. Another important question is whether autism in *TMLHE* deficient individuals can be reversed in very young children or prevented altogether. Carnitine supplementation can and should be explored promptly in young children diagnosed simultaneously with autism and *TMLHE* deficiency. Furthermore, as γ-BB levels are severely reduced in *TMLHE* deficient individuals, it will be interesting to explore whether the deficiency of γ-BB plays a role in the etiology of autism by influencing GABAergic neurotransmission. Thus, if *TMLHE* deficiency leads to the development of ASD, due to decreased carnitine or γ-BB levels during development, supplementation with these compounds might be beneficial. Unfortunately, toxicity of TML (although unlikely), deficiency of other carnitine biosynthesis intermediates, or some unknown moonlighting function of the trimethyllysine dioxygenase enzyme might prevent beneficial effects of the treatment of this disorder with carnitine or carnitine percursors.

As demonstrated by the several important queries raised and explored throughout this thesis, the investigation of carnitine function, metabolism and its derivatives remains a stimulating area, with the promise of new advances in the future.

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2. Violante S, IJlst L, te Brinke H, Tavares de Almeida I, Houten S, Ventura FV, Wanders RJ. Mitochondrial fatty acid beta-oxidation disorders: Etiology of acylcarnitines. VIII International Symposium of SPDM, Porto, 2011.

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CURRICULUM VITAE

Sara Liliana Nunes Violante was born on the 21st of June of 1983 in Lisbon, Portugal. She has graduated in Biochemistry at the Faculty of Sciences of the University of Lisbon in 2006, after research training at the Faculty of Pharmacy of the University of Lisbon (FFUL), supervised by Prof. Maria de Fátima Vieira Ventura.

After graduation, she joined the *Instituto Gulbenkian de Ciência* to integrate the Human Genetics group led by Dr. Sofia Oliveira for a 9 month professional internship. She participated in the projects "Proteomics in Parkinson disease" (Fellowship from the Duke University) and "MicroRnomics and proteomics in Parkinson disease" (PTDC/SAU-GMG/64428/2006), in collaboration with several Portuguese hospitals.

In 2007 she was granted a PhD fellowship (SFRH/BD/38074/2007) by Fundação para a Ciência e Tecnologia (FCT). The research project was supervised by Prof. Maria de Fátima Vieira Ventura and co-supervised by Prof. Ronald J. A. Wanders and Prof. Maria Isabel Ginestal Tavares de Almeida. The studies presented in this thesis entitled "Novel Aspects of Carnitine Function and Metabolism" were performed at two host institutions: the Research Institute for Medicines and Pharmaceutical Sciences – *i*Med.UL, FFUL, Portugal and the Laboratory of Genetic Metabolic Diseases, Department of Clinical Chemistry and Pediatrics, Academic Medical Centre, University of Amsterdam, The Netherlands. During the development of the studies that incorporate this thesis, several oral and poster communications were presented.

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