HEINRICH HEINE
UNIVERSITÄT
DÜSSELDORF

Carnitine homeostasis and dietary modification in long-chain fatty acid oxidation disorders

Inaugural-Dissertation

zur Erlangung des Doktorgrades
der Mathematisch-Naturwissenschaftlichen Fakultät
der Heinrich-Heine-Universität Düsseldorf

vorgelegt von

Sonja Primaßin

aus Krefeld

Düsseldorf, Juli 2010

aus der Klinik für Allgemeine Pädiatrie des Universitätsklinikums
der Heinrich-Heine Universität Düsseldorf

Gedruckt mit der Genehmigung der
Mathematisch-Naturwissenschaftlichen Fakultät der
Heinrich-Heine-Universität Düsseldorf

Referent: Prof. Dr. Ute Spiekerkötter
Koreferent: Prof. Dr. Jörg Breitkreutz

Tag der mündlichen Prüfung: 07.09.2010

Table of contents

List of abbreviations

γ-BB	γ-butyrobetaine
γ-BBD	γ-butyrobetaine dioxygenase
ACAD	acyl-CoA dehydrogenase
ACC-1α	acyl-CoA carboxylase
ATP	adenosine triphosphate
BSA	bovine serum albumine
BCA	bicinchoninic acid
CACT	carnitine/acylcarnitine translocase
CAT	carnitine acetyltranferase
CoA	coenzyme A
CoASH	free coenzyme A
COT	carnitine octanoyltransferase
CPT-I	carnitine palmitoyltransferase I
CPT-II	carnitine palmitoyltransferase II
DBS	dried blood spot
ESI	electrospray ionization
FAOD	fatty acid oxidation disorder
FASN	fatty acid synthase
FFA	free fatty acids
GA-I	glutaric aciduria I
HPLC	high performance liquid chromatography
HTML	3-hydroxy-6-N-trimethyllysine
IVA	isovaleric aciduria
KO	knock-out
LCAD	long-chain acyl-CoA dehydrogenase
LCHAD	long-chain 3-hydroxyl-acyl-CoA dehydrogenase
LCHYD	long-chain enoyl-CoA hydratase
LCT	long-chain triglycerides
MA	malonic aciduria
MAD	multiple acyl-CoA dehydrogenase
MCAD	medium-chain acyl-CoA dehydrogenase
MCFA	medium-chain fatty acids

MCT	medium-chain triglycerides
MMA	methylmalonic aciduria
MS	mass spectrometry
MS/MS	tandem mass spectrometry
NBS	newborn screening
OA	organic aciduria
PA	propionic aciduria
SCAD	short-chain acyl-CoA dehydrogenase
SCD1	stearoyl-Coenzyme A desaturase
SCHAD	short-chain 3-hydroxyl-acyl-CoA dehydrogenase
SCHYD	short-chain enoyl-CoA hydratase
SEM	standard error of the mean
SPE	solid-phase extraction
SREBP-1c	sterol regulatory element binding transcription factor
TFP	trifunctional protein
TGA	triglyceride
TMABA	4-trimethylaminobutyraldehyde
TML	6-N-trimethyllysine
VLCAD	very long-chain acyl-CoA dehydrogenase
VLCADD	very long-chain acyl-CoA dehydrogenase deficiency
VLCAD KO	very long-chain acyl-CoA dehydrogenase knock-out
WT	wild-type

Chapter 1

Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD) is the first enzyme in the mitochondrial β-oxidation cycle, which plays an important role in human long-chain fatty acid degradation. Deficiency of VLCAD may present as neonatal-onset life-threatening metabolic derangement or with milder later-onset phenotypes. Newborn screening (NBS) using modern diagnostic investigation methods, i.e. electrospray ionization tandem mass spectrometry (ESI-MS/MS), identifies fatty acid oxidation defects in the first days of life. So far, it is still unknown, which of the three factors: environmental stressors, genotype or residual enzyme activity mainly influences pathophysiology. Moreover, since some defects remain life-threatening diseases despite NBS, current treatment measures need improvement. Blood is the preferred parameter for clinical monitoring, as tissue samples are difficult to obtain in infantile patients.

1.1 Fatty acid metabolism

Living beings need energy and matter for living. Therefore they must have an unceasing supply of energy and matter. The transformation of this energy and matter within the body is called metabolism. Metabolism is composed of catabolism and anabolism. Typically, in catabolism, larger organic molecules are broken down into smaller constituents. This generally occurs with the release of energy, typically as adenosine triphosphate (ATP). During anabolism small precursor molecules are assembled into larger organic molecules. This always requires the input of energy, often as ATP. Anabolism and catabolism are always aiming for a balance. The free energy, bound in carbohydrates, lipids and proteins is stored either in form of ATP or in reduced nicotinamide adenine dinucleotide phosphate (NADPH) and reduced flavin adenine nucleotide ($FADH_2$).

Although different metabolic pathways for the many variations of substrate assembling and disassembling exist, the number of intermediates is limited to a few. Acetyl-coenzyme A (CoA) plays a central role as a combined intermediate of carbohydrate, protein and lipid metabolism. The formed acetyl-CoA is oxidized through enzymatic reactions of the citric acid cycle and oxidative phosphorylation into CO_2 and H_2O. Alongside glycolytic degradation of carbohydrates, their further oxidation in the citric acid cycle and oxidative phosphorylation, fatty acids and their intramitochondrial degradation play an important role (Bartlett and Eaton 2004). Fatty acids, stored as complex lipids, are an important source of energy, especially in times of increased energy demand or depleted carbohydrate stores, because fatty acids are both reduced and anhydrous. They are stored in the cytoplasm of many cells, mostly adipocytes, in form of triglyceride droplets. Fatty acids have nearly twice the energy output as

carbohydrates and proteins because of their low oxidation state. The energy yielded from a gram of fatty acids is approximately 9 kcal compared to 4 kcal from carbohydrates.

Long-chain fatty acids are ingested as triglycerides, which cannot be absorbed by the intestine. They are, therefore, hydrolyzed into free fatty acids and monoglycerides by pancreatic lipase. Once across the intestinal barrier, again condensed as triglycerides and reassembled into chylomicrons or liposomes, they are secreted into the lacteals, the capillaries of the lymph system, and subsequently into the blood. Eventually, they bind to the membranes of hepatocytes, adipocytes or muscle fibers, where they are either stored or oxidized for energy production. The liver acts as a major organ for fatty acid metabolism processing chylomicron remnants and liposomes into various lipoprotein forms. Fatty acid transport proteins (FATPs) are integral transmembrane proteins that enhance the uptake of long-chain and very long-chain fatty acids into cells (Doege and Stahl 2006). Besides FATPs, heart and skeletal muscle cells also possess plasma membrane fatty acid binding proteins (FABPs) and fatty acid translocase (FAT, CD36), both involved in the uptake of fatty acids, in order to maintain high rates of fatty acid oxidation (Matarese et al. 1989; Veerkamp et al. 1991).

1.2 Mitochondrial β-oxidation

Already in 1904 the German biochemist Georg Franz Knoop demonstrated, that oxidation occurs at the C-terminal end during fatty acid degradation (Knoop 1904).

Mitochondrial β-oxidation is the key metabolic pathway for energy homeostasis in periods of higher energy demand such as fasting and prolonged exercise. During this process fatty acids are completely oxidized to acetyl-CoA. The synthesis of energy includes three major steps: Activation of fatty acids and transport into mitochondria, β-oxidation itself and oxidative phosphorylation (Bartlett and Eaton 2004; Rinaldo et al. 2002). After entering the cell, fatty acids are activated through esterification with CoA. The activation is ATP-dependent and it is catalyzed by several chain-length specific acyl-CoA-synthetases localized in different cell compartments (Watkins et al. 2007), which all follow this formula:

$$\text{R-COOH} + \text{ATP} + \text{CoA–SH} \rightarrow \text{R-CO-SCoA} + \text{AMP} + \text{PPi}$$

Short-chain fatty acids are activated in the cytosol, medium-chain fatty acids are activated in the mitochondrial matrix by *medium-chain acyl-CoA synthetase* and long-chain fatty acids by *long-chain acyl-CoA synthetase* located in the outer mitochondrial membrane (Schulz 1994).

Short- and medium-chain fatty acids can pass the outer and inner mitochondrial membranes by diffusion. In contrast, long-chain fatty acids are transported as acylcarnitines across the outer mitochondrial membrane (see 1.4.1). During the oxidation process acyl-CoAs are degraded into acetyl-CoA, which are repeatedly cleaved from the fatty acid via a 4-step cycle, until the fatty acid is completely degraded (Figure 1-1). First, acyl-CoA is oxidized by acyl-CoA dehydrogenase to yield a trans-2-enoyl-CoA. This step is followed by hydratation of the double bond. The resulting L-3-hydroxy-acyl-CoA is again oxidized into 3-keto-acyl-CoA in the third step. Finally, the thiolytic cleavage of 3-keto-acyl-CoA produces a 2-carbon chain-shortened acyl-CoA plus acetyl-CoA. Each enzyme involved in the process is chain-length specific. The cleaved acetyl-CoAs can either enter the citric acid cycle, which produces NADH and $FADH_2$, or they can be used for the synthesis of ketone bodies in the liver and kidney (Bartlett and Eaton 2004; Rinaldo et al. 2002). NADH and $FADH_2$ are subsequently used in the electron transport chain to produce ATP. Oxidation of very-long fatty acids (>20 carbons) to long-chain fatty acids (≤18 carbons) occurs in peroxisomes.

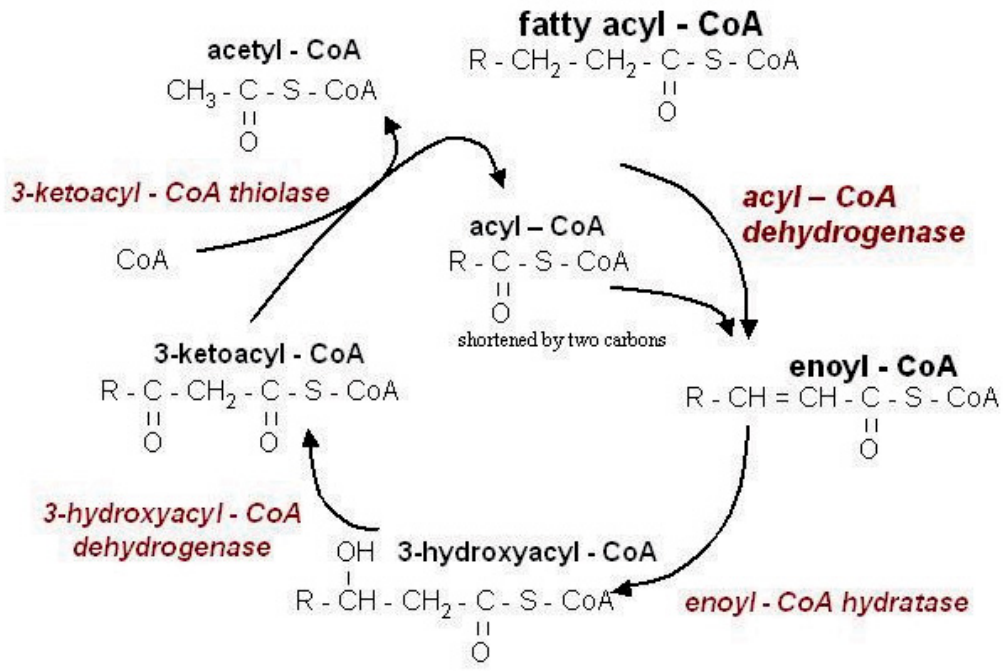


Figure 1-1: Mitochondrial β-oxidation of fatty acids, modified from Vockley et al. (Vockley et al. 2002).

The first step in the mitochondrial β-oxidation cycle is catalyzed either by short-chain acyl-CoA dehydrogenase (SCAD, for C4-C6 acyl-CoAs), medium-chain acyl-CoA dehydrogenase (MCAD, for C6-C10 acyl-CoAs), or very long-chain acyl-CoA dehydrogenase (VLCAD, for C12-C24 acyl-CoAs) (Eaton et al. 1996). Long-chain acyl-CoA

dehydrogenase (LCAD), whose preferred substrate chain length is between C8-C18 acyl-CoAs, is also part of this family, however, its specific role in mitochondrial β-oxidation in humans remains unclear, since deficiency of this enzyme has never been reported. Acyl-CoA dehydrogenase 9 (ACAD9) has only recently been described as another enzyme responsible for the β-oxidation of unsaturated fatty acids (Ensenauer et al. 2005).

The enzymes medium-/short-chain enoyl-CoA hydratase (M/SCHYD), also called crotonase, and medium-/short-chain 3-hydroxy-acyl-CoA dehydrogenase (M/SCHAD) have activities towards fatty acids with a chain-length up to C10. The enzymes long-chain enoyl-CoA hydratase (LCHYD) and long-chain 3-hydroxy-acyl-CoA dehydrogenase (LCHAD) are responsible for fatty acids with a chain length of C8 and upwards (Bartlett and Eaton 2004; Rinaldo et al. 2002; Uchida et al. 1992).

1.3 Fatty acid oxidation disorders

Disorders of fatty acid oxidation are either caused by defects of mitochondrial beta-oxidation enzymes or by enzymes of the carnitine-bound transport of fatty acids via the mitochondrial membranes (Wanders et al. 1999). Generally these defects are inherited in an autosomal recessive fashion with a combined incidence of 1:9.300 (Lindner et al. 2010). The first described fatty acid oxidation disorder was carnitine palmitoyltransferase II (CPT-II) deficiency in 1973, followed by CPT-I in 1975 and MCAD deficiency in 1976 (DiMauro and DiMauro 1973; Gregersen et al. 1976; Karpati et al. 1975). Only recently ACAD9-deficiency has been identified (Ensenauer et al. 2005). Hence, inborn fatty acid oxidation disorders are important diseases for research with respect to diagnosis and treatment.

Today almost 25 defects of fatty acid oxidation are known and many of them become manifest in early childhood with life-threatening metabolic crisis, due to energy deficiency and accumulation of non-metabolizable fatty acids and their derivates in tissues (Bennett et al. 2000; Saudubray et al. 1999). Accumulating metabolites may have toxic effects on different organ systems such as brain, skeletal muscle, heart or liver. Therefore, heterogeneous clinical symptoms occur such as hypoketotic hypoglycemia and coma, cardiomyopathy, skeletal myopathy, and exercise-dependent rhabdomyolysis (Vockley et al. 2002).

1.3.1 VLCAD deficiency

Deficiency of VLCAD is the most common mitochondrial β-oxidation defect of long-chain fatty acids, with an occurence of ~1:50.000 to 1:100.000 births (Spiekerkoetter et al. 2003). In humans, VLCAD deficiency is characterized by phenotypic heterogeneity as most

of the long-chain fatty acid oxidation defects. Since implementation of NBS for fatty acid oxidation defects, VLCAD-deficient patients mostly appear asymptomatic at birth, but in catabolic situations, when lipolysis is induced, a previously healthy child may become critically ill without preventive measures. Different forms of presentation are distinguished: a severe early onset form presenting with cardiomyopathy and Reye-like symptoms; a hepatic phenotype that usually expresses in infancy with recurrent hypoketotic hypoglycemia; and a milder, later-onset, myopathic form with episodic muscle weakness and rhabdomyolysis (Gregersen et al. 2004). However, the hepatic phenotype of infancy may turn into a muscular phenotype during childhood and adolescence. NBS programs for fatty acid oxidation defects have significantly reduced disease morbidity and mortality. However, it is unknown whether initially asymptomatic patients may remain asymptomatic throughout life.

1.3.2 Diagnosis and treatment

The primary diagnostic tool for fatty acid oxidation defects today is the determination of free carnitine and acylcarnitines by ESI-MS/MS (Rinaldo et al. 2002). Blood serum, plasma, urine or dried blood spot (DBS) samples can be used for extraction and analysis of metabolites. The specific enzyme defect can generally be identified on the basis of a disease-specific metabolic profile (Chace et al. 1999a; Chace et al. 1999b; Green and Pollitt 1999). However, diagnosis always needs to be confirmed by enzyme analysis in skin fibroblasts, lymphocytes or tissues. Molecular genetic analysis is also a valid and available method for confirmation diagnosis of fatty acid oxidation disorders, although, due to molecular heterogeneity, it is much more time-consuming, more expensive and sometimes not straight-forward, in case only one mutation can be found.

Dietary fat mainly consists of long-chain fatty acids with C16-C20 chain-lengths. Because the first step of the β-oxidation cycle cannot be performed in VLCAD deficiency, long-chain acyl-CoAs accumulate in mitochondria. To leave the mitochondria, they are reconverted into acylcarnitines, which can be assayed in blood. Therefore VLCAD-deficient patients accumulate long-chain acylcarnitines, due to insufficient oxidation of long-chain acyl-CoAs (Zytkovicz et al. 2001). C14:1-carnitine is the disease-specific marker for VLCAD deficiency and is used also during newborn screening. As a result of an increased production of acylcarnitines, blood free carnitine concentrations may decrease.

Modification of dietary fat intake and frequency of meals is the main focus of current clinical practice (Vockley et al. 2002). One simple preventive measure is to ensure a constant supply of glucose by avoidance of fasting through increased frequency of meals. Fat-reduced

and fat-modified diets may avoid accumulation of long-chain acyl-CoAs and long-chain acylcarnitines (Kerner and Hoppel 1998). Medium-chain triglycerides (MCT) are structured triglycerides containing primarily medium-chain, saturated fatty acids such as C8 and C10. In contrast to long-chain triglycerides (LCT), MCT can be metabolized by VLCAD-deficient patients. Absorption and transport of MCT into the cells is also assumed to be less complicated, because MCT does not require esterification into acylcarnitines to cross mitochondrial membranes and can be more rapidly oxidized (Marten et al. 2006). This makes MCT also part of dietary treatment recommendations in disorders of the carnitine-bound transport of long-chain fatty acids through the mitochondrial membranes. However, the optimal time of application and dosage is not well established.

Also carnitine supplementation has long been used to replenish low carnitine levels and to accelerate the removal of toxic fatty acid intermediates. Its use, however, remains controversial (Spiekerkoetter et al. 2000; Touma et al. 2001; Walter 2003; Winter 2003).

1.4 Carnitine and carnitine homeostasis

Carnitine (3-hydroxy-4-N-trimethylaminobutyrate) is a small (molecular weight = 161.2 g/mol), water-soluble, quaternary nitrogen-containing molecule that is present in both L- and D- forms, with L-carnitine being the biologically active form (Figure 1-2). It is present in most, if not all, animal species and in several microorganisms and plants. In mammals, carnitine is an essential metabolite in energy metabolism, because it enables long-chain acyl compounds to cross the inner mitochondrial membrane as acylcarnitines, as only inside the mitochondria fatty acids can be further metabolized by β-oxidation. The body receives carnitine from dietary sources or from hepatic endogenous synthesis. In addition, it is efficiently reabsorbed by the kidney. All three factors are maintaining carnitine homeostasis (Vaz and Wanders 2002).

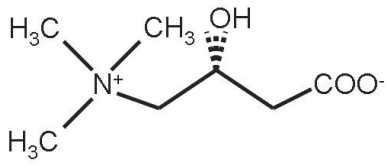


Figure 1-2: The structural formula of L-carnitine.

1.4.1 Function

Carnitine can be reversibly acylated with acetyl-CoA and stimulates fatty acid oxidation in liver homogenates (FRIEDMAN and FRAENKEL 1955; FRITZ 1955). Carnitine is substrate for reactions catalyzed by carnitine acyltransferases that convert acyl-CoA into the corresponding acylcarnitine, according to the following reversible reaction:

$$\text{Acyl-CoA} + \text{Carnitine} \leftrightarrow \text{Acylcarnitine} + \text{CoASH}$$

Carnitine is involved in the transport of activated long-chain fatty acids from the cytosol to the mitochondrial matrix, where β-oxidation takes place (Figures 1-3). Cytosolic long-chain fatty acids, which are present as CoA esters, are transesterified to L-carnitine in a reaction catalysed by CPT-I at the mitochondrial outer membrane. In this reaction, the acyl-moiety of the long-chain fatty acids is transferred from CoA to the hydroxyl group of carnitine (McGarry and Brown 1997). The resulting long-chain acyl-carnitine esters are transported over the inner mitochondrial membrane via a specific carrier, carnitine acyl-carnitine translocase (CACT; Pande 1975). At the matrix side of the mitochondrial membrane the long-chain fatty acids are transesterified to intramitochondrial CoA, a reaction catalysed by carnitine CPT-II. The released carnitine leaves the mitochondria into the cytosol via CACT for a subsequent transport (McGarry and Brown 1997). Carnitine can also be converted into an acylcarnitine by CPT-II or carnitine acetyltransferase (CAT) in case of accumulation of acyl-CoA and can then be exported from the mitochondrial matrix, out of the cell, followed by excretion from the body via either urine or bile (Ramsay et al. 2001). In the mitochondrial matrix, the enzyme CAT is also able to reconvert short- and medium-chain acyl-CoAs into acyl-carnitines using intramitochondrial carnitine. This has been confirmed in mitochondria, peroxisomes and microsomes (Edwards et al. 1974; Kahonen 1976). Through the action of acyltransferases, carnitine and acyl-carnitines are rapidly interconvertible (Brass and Hoppel 1980a; Bremer 1983).

Three different transferases have been described, distinguished by their substrate specificity, their cellular localization, their structure and reactivity with inhibitors (Bremer 1983; Solberg 1974). CPT-I and CPT-II use long-chain acyl-groups as substrates and are localized in mitochondria (Miyazawa et al. 1983). Carnitine octanoyltransferase (COT) utilizes medium-chain acyl-moiety as substrate and is localized in peroxisomes and microsomes (Miyazawa et al. 1983).

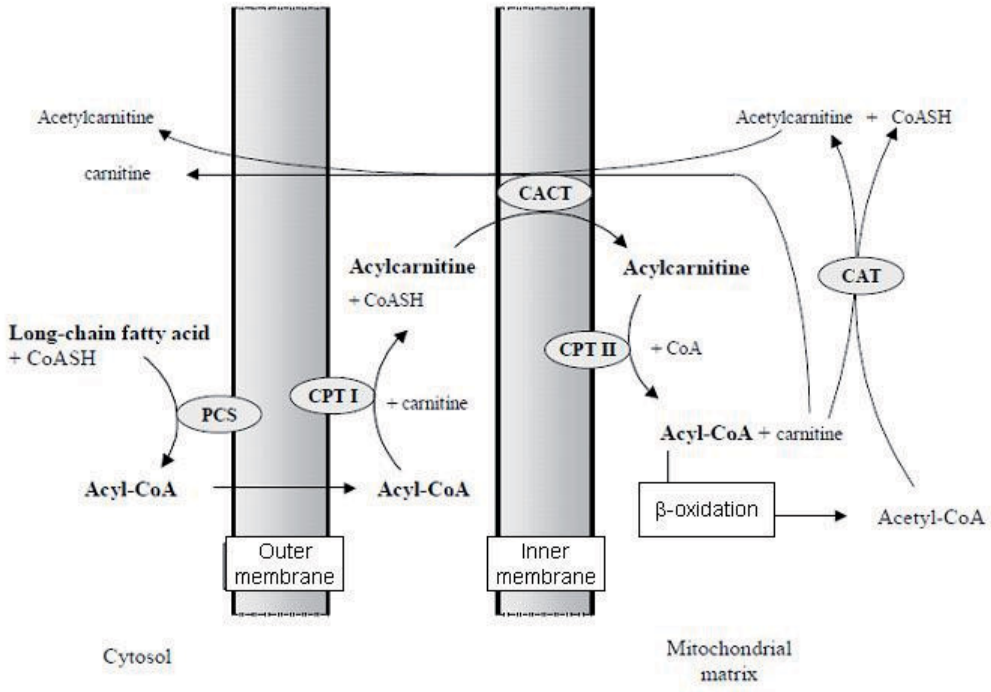


Figure 1-3: Transport of activated fatty acids into mitochondria, modified from Vaz and Wanders (Vaz and Wanders 2002).

1.4.2 Buffering of the mitochondrial acyl-CoA/CoA ratio

In the mitochondrial matrix, the enzyme CAT converts short-chain acyl-CoAs into the corresponding acylcarnitines by using mitochondrial carnitine and releasing free CoA (CoASH; Bieber 1988; Brass and Hoppel 1980b). Through the action of CAT, the carnitine and CoA pools are in close correlation. Experiments performed with rat heart (Pearson and Tubbs 1967), frog muscle (Alkonyi et al. 1975), sheep liver (Snoswell and Henderson 1970) and human skeletal muscle (Friolet et al. 1994) indicated that carnitine acts as a buffer for free CoA. Brass and Hoppel (Brass and Hoppel 1980b) demonstrated that in rat liver and under different metabolic conditions, the L-carnitine pool reflects the CoA pool. This reversible exchange allows the cell to regulate its levels of free CoA using carnitine as a buffer. Since carnitine is in most tissues in a much higher concentration available than free CoA, the extramitochondrial acetylcarnitine/carnitine ratio will prevent great fluctuations in the mitochondrial acetyl-CoA/CoA ratio by formation of acyl-CoA (Bremer 1983; Ramsay et al. 2001).

1.4.3 Removal of potentially toxic acyl-groups

The buffering effect of carnitine can be expanded to the regulation of poorly metabolized and potentially toxic acyl-groups, resulting either from xenobiotics, e.g. pivalic acid and valproate (Arrigoni-Martelli and Caso 2001), or from blockage of a normal metabolic pathway (e.g. propionic acid in propionic acidemia; Rebouche and Seim 1998). These acyl-groups are converted to CoA-derivatives, thus depleting the cellular pool of free CoA. The subsequent transesterification of these acyl-CoAs to the corresponding acylcarnitines, followed by their excretion in the urine (Duran et al. 1990; Melegh et al. 1987), replenish the free CoA pool, however, they lead to secondary carnitine deficiency that is assumed to be replenished by carnitine supplementation (Holme et al. 1992; Melegh et al. 1993).

1.4.4 Biosynthesis

Apart from dietary intake of carnitine, most eukaryotes are able to synthesize carnitine from 6-N-trimethyllysine (TML; Bremer 1983; Kaufman and Broquist 1977; Lindstedt and Lindstedt 1970). TML is generated by the hydrolysis of proteins containing lysines that are trimethylated at their ε-amino group by a protein-dependent methyltransferase using S-adenosyl methionine as methyl donor (Paik and Kim 1971). TML is then hydroxylated by the enzyme TML-dioxygenase to 3-hydroxy-6-N-trimethyllysine (HTML). HTML is afterwards cleaved by a specific aldolase to yield 4-trimethylaminobutyraldehyde (TMABA), which is oxidized to γ-butyrobetaine (4-trimethylaminobutyric acid) by TMABA-dehydrogenase. γ-butyrobetaine is the last intermediate in the carnitine biosynthesis pathway and is hydroxylated in the liver by γ-butyrobetaine dioxygenase (EC 1.14.11.1) to yield free carnitine (Figure 1-4). Nevertheless γ-butyrobetaine and its presursors in the carnitine biosynthesis pathway have been detected in various tissues like muscle, heart and kidney (van Vlies et al. 2005). Moreover, all the enzymes forming these precursors were present in these tissues except γ-butyrobetaine dioxygenase (van Vlies et al. 2006). The latter enzyme is only present in human liver, kidney and brain in humans demonstrating that only in these organs full endogenous carnitine biosynthesis can be performed (Vaz and Wanders 2002).

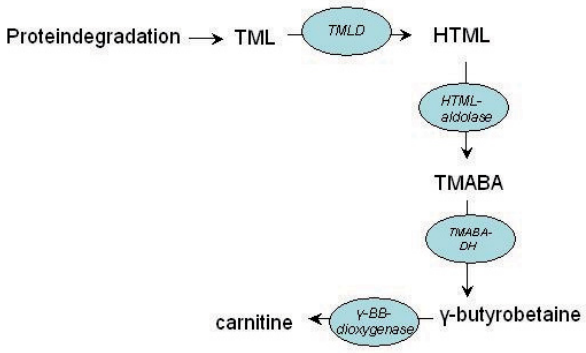


Figure 1-4: The carnitine biosynthesis pathway, adapted from Vaz and Wanders (Vaz and Wanders 2002)

1.5 Mouse model for VLCAD deficiency

In the late 1990s two mouse models for human VLCAD deficiency were generated (Exil et al. 2003; Schuler and Wood 2002). The VLCAD knockout (KO) mouse is viable and demonstrates no VLCAD-specific clinical phenotype under resting conditions in the first 6 months of life besides an increased fat storage in tissues (Exil et al. 2003). Physical exercise or fasting, however, induce similar clinical phenotypes to human VLCAD deficiency. VLCAD KO mice present with stress-induced hypoglycemia, skeletal myopathy and cold intolerance associated with elevated C14-C18 acylcarnitines (Spiekerkoetter et al. 2005). Overall, the VLCAD KO mouse is an excellent model to study human VLCAD deficiency. Kurtz et al. (Kurtz et al. 1998) also developed an LCAD KO mouse presenting rather severe symptoms of cardiomyopathy, hypoketotic hypoglycemia and unprovoked sudden death. Nevertheless LCAD deficiency has so far not been diagnosed in humans, probably due to minor expression of LCAD (Chegary et al. 2009).

1.6 Aim and outline of this thesis

Since VLCAD deficiency has been part of NBS programs, many patients remain asymptomatic with a variety of preventive measures. If symptoms occur, e.g. during catabolism, they are generally reversible with such measures. Nevertheless the exact pathomechanims responsible for the heterogeneous symptoms are still unclear. Discovering those would lay the basis for new therapeutical interventions.

Carnitine plays a major role in long-chain fatty acid oxidation and regulation of carnitine homeostasis is important to study, preferably in the VLCAD KO mouse. In Chapter 2 we investigated the effects of carnitine supplementation on different organs. The main question was whether carnitine supplementation can prevent secondary carnitine deficiency. In

addition the effects of carnitine on acylcarnitine production were studied. Also the relation between free carnitine and γ-butyrobetaine levels in tissues was identified.

In Chapter 3 we investigated which blood compartment most reliably reflects the carnitine status in patients with fatty acid oxidation disorders (FAODs) and whether γ-butyrobetaine can be used as a blood marker for induced carnitine biosynthesis.

Patients with fatty acid oxidation defects are today identified by NBS using ESI-MS/MS. However, the acylcarnitine profile is not always indicative of a definite diagnosis. In addition with heterogenous clinical phenotypes of different severity, there is also the request to predict the expected phenotype from the biochemical markers available. Therefore, we developed an enzyme assay to determine residual enzyme activity, which can be used for rapid confirmation diagnosis in newborns and possibly allows identification of the expected severity of the clinical phenotype (Chapter 4).

One important treatment measure in long-chain FAODs is the administration of MCT to disable accumulation of long-chain fatty acids by avoiding the deficient pathway. In Chapter 5 we investigated how different MCT administrations affect carnitine homeostasis and energy supply in different organs of VLCAD KO mice.

Chapter 6 demonstrates how MCT are processed in the liver of VLCAD KO mice and how a long-term MCT diet affects the overall fat metabolism.

In order to define the importance of an LCT restriction and fat modification as dietary measures in case of asymptomatic or symptomatic VLCAD deficiency, we aimed to determine in Chapter 7 how different fat-modified diets affect carnitine homeostasis and lipid metabolism .

For the study of the relation between acyl-CoA compounds and acylcarnitine compounds in tissues, we developed a method to measure both compounds within the same sample (Chapter 8).

References

1. Alkonyi I, Kerner J, Sandor A (1975) The possible role of carnitine and carnitine acetyl-transferase in the contracting frog skeletal muscle. FEBS Lett 52:265-268

2. Arrigoni-Martelli E, Caso V (2001) Carnitine protects mitochondria and removes toxic acyls from xenobiotics. Drugs Exp Clin Res 27:27-49

3. Bartlett K, Eaton S (2004) Mitochondrial beta-oxidation. Eur J Biochem 271:462-469

4. Bennett MJ, Rinaldo P, Strauss AW (2000) Inborn errors of mitochondrial fatty acid oxidation. Crit Rev Clin Lab Sci 37:1-44

5. Bieber LL (1988) Carnitine. Annu Rev Biochem 57:261-283

6. Brass EP, Hoppel CL (1980a) Effect of carnitine on mitochondrial oxidation of palmitoylearnitine. Biochem J 188:451-458

7. Brass EP, Hoppel CL (1980b) Relationship between acid-soluble carnitine and coenzyme A pools in vivo. Biochem J 190:495-504

8. Bremer J (1983) Carnitine--metabolism and functions. Physiol Rev 63:1420-1480

9. Chace DH, Adam BW, Smith SJ, Alexander JR, Hillman SL, Hannon WH (1999a) Validation of accuracy-based amino acid reference materials in dried-blood spots by tandem mass spectrometry for newborn screening assays. Clin Chem 45:1269-1277

10. Chace DH, DiPerna JC, Naylor EW (1999b) Laboratory integration and utilization of tandem mass spectrometry in neonatal screening: a model for clinical mass spectrometry in the next millennium. Acta Paediatr Suppl 88:45-47

11. Chegary M, Brinke HT, Ruiter JP, Wijburg FA, Stoll MS, Minkler PE, van WM, Schulz H, Hoppel CL, Wanders RJ, Houten SM (2009) Mitochondrial long chain fatty acid beta-oxidation in man and mouse. Biochim Biophys Acta 1791:806-815

12. DiMauro S, DiMauro PM (1973) Muscle carnitine palmityltransferase deficiency and myoglobinuria. Science 182:929-931

13. Doege H, Stahl A (2006) Protein-mediated fatty acid uptake: novel insights from in vivo models. Physiology (Bethesda) 21:259-268

14. Duran M, Loof NE, Ketting D, Dorland L (1990) Secondary carnitine deficiency. J Clin Chem Clin Biochem 28:359-363

15. Eaton S, Bartlett K, Pourfarzam M (1996) Mammalian mitochondrial beta-oxidation. Biochem J 320 (Pt 2):345-357

16. Edwards YH, Chase JF, Edwards MR, Tubbs PK (1974) Carnitine acetyltransferase: the question of multiple forms. Eur J Biochem 46:209-215

17. Ensenauer R, He M, Willard JM, Goetzman ES, Corydon TJ, Vandahl BB, Mohsen AW, Isaya G, Vockley J (2005) Human acyl-CoA dehydrogenase-9 plays a novel role in

the mitochondrial beta-oxidation of unsaturated fatty acids. J Biol Chem 280:32309-32316

18. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

19. FRIEDMAN S, FRAENKEL G (1955) Reversible enzymatic acetylation of carnitine. Arch Biochem Biophys 59:491-501

20. Friolet R, Hoppeler H, Krahenbuhl S (1994) Relationship between the coenzyme A and the carnitine pools in human skeletal muscle at rest and after exhaustive exercise under normoxic and acutely hypoxic conditions. J Clin Invest 94:1490-1495

21. FRITZ I (1955) The effect of muscle extracts on the oxidation of palmitic acid by liver slices and homogenates. Acta Physiol Scand 34:367-385

22. Green A, Pollitt RJ (1999) Population newborn screening for inherited metabolic disease: current UK perspectives. J Inherit Metab Dis 22:572-579

23. Gregersen N, Bross P, Andresen BS (2004) Genetic defects in fatty acid beta-oxidation and acyl-CoA dehydrogenases. Molecular pathogenesis and genotype-phenotype relationships. Eur J Biochem 271:470-482

24. Gregersen N, Lauritzen R, Rasmussen K (1976) Suberylglycine excretion in the urine from a patient with dicarboxylic aciduria. Clin Chim Acta 70:417-425

25. Holme E, Jodal U, Linstedt S, Nordin I (1992) Effects of pivalic acid-containing prodrugs on carnitine homeostasis and on response to fasting in children. Scand J Clin Lab Invest 52:361-372

26. Kahonen MT (1976) Effect of clofibrate treatment on carnitine acyltransferases in different subcellular fractions of rat liver. Biochim Biophys Acta 428:690-701

27. Karpati G, Carpenter S, Engel AG, Watters G, Allen J, Rothman S, Klassen G, Mamer OA (1975) The syndrome of systemic carnitine deficiency. Clinical, morphologic, biochemical, and pathophysiologic features. Neurology 25:16-24

28. Kaufman RA, Broquist HP (1977) Biosynthesis of carnitine in Neurospora crassa. J Biol Chem 252:7437-7439

29. Kerner J, Hoppel C (1998) Genetic disorders of carnitine metabolism and their nutritional management. Annu Rev Nutr 18:179-206

30. Knoop F (1904) Der Abbau aromatischer Fettsäuren im Tierkörper. Beitr Chem Physiol Pathol 6:150-162

31. Kurtz DM, Rinaldo P, Rhead WJ, Tian L, Millington DS, Vockley J, Hamm DA, Brix AE, Lindsey JR, Pinkert CA, O'Brien WE, Wood PA (1998) Targeted disruption of mouse long-chain acyl-CoA dehydrogenase gene reveals crucial roles for fatty acid oxidation. Proc Natl Acad Sci U S A 95:15592-15597

32. Lindner M, Hoffmann GF, Matern D (2010) Newborn screening for disorders of fatty-acid oxidation: experience and recommendations from an expert meeting. J Inherit Metab Dis

33. Lindstedt G, Lindstedt S (1970) Cofactor requirements of gamma-butyrobetaine hydroxylase from rat liver. J Biol Chem 245:4178-4186

34. Marten, B, Pfeuffer M, and Schrezenmeir J. Medium-chain triglycerides. 16, 1374-1382. 2-6-2006.
Ref Type: Generic

35. Matarese V, Stone RL, Waggoner DW, Bernlohr DA (1989) Intracellular fatty acid trafficking and the role of cytosolic lipid binding proteins. Prog Lipid Res 28:245-272

36. McGarry JD, Brown NF (1997) The mitochondrial carnitine palmitoyltransferase system. From concept to molecular analysis. Eur J Biochem 244:1-14

37. Melegh B, Kerner J, Bieber LL (1987) Pivampicillin-promoted excretion of pivaloylcarnitine in humans. Biochem Pharmacol 36:3405-3409

38. Melegh B, Sumegi B, Sherry AD (1993) Preferential elimination of pivalate with supplemental carnitine via formation of pivaloylcarnitine in man. Xenobiotica 23:1255-1261

39. Miyazawa S, Ozasa H, Osumi T, Hashimoto T (1983) Purification and properties of carnitine octanoyltransferase and carnitine palmitoyltransferase from rat liver. J Biochem 94:529-542

40. Paik WK, Kim S (1971) Protein methylation. Science 174:114-119

41. Pande SV (1975) A mitochondrial carnitine acylcarnitine translocase system. Proc Natl Acad Sci U S A 72:883-887

42. Pearson DJ, Tubbs PK (1967) Carnitine and derivatives in rat tissues. Biochem J 105:953-963

43. Ramsay RR, Gandour RD, van der Leij FR (2001) Molecular enzymology of carnitine transfer and transport. Biochim Biophys Acta 1546:21-43

44. Rebouche CJ, Seim H (1998) Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr 18:39-61

45. Rinaldo P, Matern D, Bennett MJ (2002) Fatty acid oxidation disorders. Annu Rev Physiol 64:477-502

46. Saudubray JM, Martin D, de LP, Touati G, Poggi-Travert F, Bonnet D, Jouvet P, Boutron M, Slama A, Vianey-Saban C, Bonnefont JP, Rabier D, Kamoun P, Brivet M (1999) Recognition and management of fatty acid oxidation defects: a series of 107 patients. J Inherit Metab Dis 22:488-502

47. Schuler AM, Wood PA (2002) Mouse models for disorders of mitochondrial fatty acid beta-oxidation. ILAR J 43:57-65

48. Schulz H (1994) Regulation of fatty acid oxidation in heart. J Nutr 124:165-171

49. Snoswell AM, Henderson GD (1970) Aspects of carnitine ester metabolism in sheep liver. Biochem J 119:59-65

50. Solberg HE (1974) Acyl group specificity of mitochondrial pools of carnitine acyltransferases. Biochim Biophys Acta 360:101-112

51. Spiekerkoetter U, Schwahn B, Korall H, Trefz FK, Andresen BS, Wendel U (2000) Very-long-chain acyl-coenzyme A dehydrogenase (VLCAD) deficiency: monitoring of treatment by carnitine/acylcarnitine analysis in blood spots. Acta Paediatr 89:492-495

52. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U (2003) MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr 143:335-342

53. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, IJlst L, Vaz FM, van VN, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

54. Touma EH, Rashed MS, Vianey-Saban C, Sakr A, Divry P, Gregersen N, Andresen BS (2001) A severe genotype with favourable outcome in very long chain acyl-CoA dehydrogenase deficiency. Arch Dis Child 84:58-60

55. Uchida Y, Izai K, Orii T, Hashimoto T (1992) Novel fatty acid beta-oxidation enzymes in rat liver mitochondria. II. Purification and properties of enoyl-coenzyme A (CoA) hydratase/3-hydroxyacyl-CoA dehydrogenase/3-ketoacyl-CoA thiolase trifunctional protein. J Biol Chem 267:1034-1041

56. van Vlies N, Wanders RJ, Vaz FM (2006) Measurement of carnitine biosynthesis enzyme activities by tandem mass spectrometry: differences between the mouse and the rat. Anal Biochem 354:132-139

57. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

58. Vaz FM, Wanders RJ (2002) Carnitine biosynthesis in mammals. Biochem J 361:417-429

59. Veerkamp JH, Peeters RA, Maatman RG (1991) Structural and functional features of different types of cytoplasmic fatty acid-binding proteins. Biochim Biophys Acta 1081:1-24

60. Vockley J, Singh RH, Whiteman DA (2002) Diagnosis and management of defects of mitochondrial beta-oxidation. Curr Opin Clin Nutr Metab Care 5:601-609

61. Walter JH (2003) L-carnitine in inborn errors of metabolism: what is the evidence? J Inherit Metab Dis 26:181-188

62. Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, Ijlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. J Inherit Metab Dis 22:442-487

63. Watkins PA, Maiguel D, Jia Z, Pevsner J (2007) Evidence for 26 distinct acyl-coenzyme A synthetase genes in the human genome. J Lipid Res 48:2736-2750

64. Winter SC (2003) Treatment of carnitine deficiency. J Inherit Metab Dis 26:171-180

65. Zytkovicz TH, Fitzgerald EF, Marsden D, Larson CA, Shih VE, Johnson DM, Strauss AW, Comeau AM, Eaton RB, Grady GF (2001) Tandem mass spectrometric analysis for amino, organic, and fatty acid disorders in newborn dried blood spots: a two-year summary from the New England Newborn Screening Program. Clin Chem 47:1945-1955

Chapter 2

Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine

S. Primassin, F. ter Veld, E. Mayatepek and U. Spiekerkoetter

Pediatr Res. **2008;63(6): 632–7**

Abstract

Background

Deficiency of very long-chain acyl-CoA dehydrogenase (VLCAD) results in accumulation of C14-C18 acylcarnitines and low free carnitine. Carnitine supplementation is still controversial. VLCAD knock-out (KO) mice exhibit a similar clinical and biochemical phenotype to those observed in humans.

Methods

VLCAD KO mice were fed with carnitine dissolved in drinking water. Carnitine, acylcarnitines and γ-butyrobetaine were measured in blood and tissues. Measurements were performed under resting conditions, after exercise and after 24 hours of regeneration. HepG2 cells were incubated with palmitoyl-CoA and palmitoyl-carnitine, respectively, in order to examine toxicity.

Results

With carnitine supplementation, acylcarnitine production was significantly induced. Nevertheless, carnitine was low in skeletal muscle after exercise. Without carnitine supplementation, liver carnitine significantly increased following exercise, and after 24 hours of regeneration, carnitine concentrations in skeletal muscle completely replenished to initial values. Incubation of hepatic cells with palmitoyl-CoA and palmitoyl-carnitine revealed significantly reduced cell viability after incubation with palmitoyl-carnitine.

Conclusion

The present study demonstrates that carnitine supplementation results in significant accumulation of potentially toxic acylcarnitines in tissues. The expected prevention of low tissue carnitine was not confirmed. The principle mechanism regulating carnitine homeostasis appears to be endogenous carnitine biosynthesis, also under conditions with increased demand of carnitine such as in VLCAD deficiency.

Introduction

Very long-chain acyl-CoA dehydrogenase (VLCAD or ACADVL, EC 1.3.99.3) is one of several enzymes of mitochondrial β-oxidation. Deficiency of VLCAD is the most common mitochondrial β-oxidation defect of long-chain fatty acids, with an occurence of ~1:50.000 to 1:100.000 births (Spiekerkoetter et al. 2003). In humans, VLCAD deficiency is characterized by phenotypic heterogeneity. Phenotypic presentation is heterogeneous and different forms of

presentation are distinguished: a severe early onset form presenting with cardiomyopathy and Reye-like symptoms; a hepatic phenotype that usually expresses in infancy with recurrent hypoketotic hypoglycemia; and a milder, later-onset, myopathic form with episodic muscle weakness and rhabdomyolysis (Gregersen et al. 2004). However, the hepatic phenotype of infancy will often become a muscular phenotype during childhood and adolescence. Exercise or catabolic stress such as illness triggers clinical symptoms. With the start of neonatal screening programs for fatty acid oxidation defects the majority of patients are asymptomatic at time of diagnosis and remain asymptomatic with preventive measures during the first years of follow-up. Especially for this group of patients, there is a need to define risk factors for the manifestation of clinical symptoms with special respect to physical exercise. Deficient oxidation of long-chain acyl-CoAs, especially during catabolism, results in accumulation of long-chain acylcarnitines.

Carnitine is an essential metabolite in energy metabolism, because it enables long-chain acyl compounds to cross the inner mitochondrial membrane as acylcarnitines. Only inside the mitochondria fatty acids can be metabolized by β-oxidation. The body receives carnitine from dietary sources or from endogenous synthesis. In addition, it is efficiently reabsorped by the kidney. All three factors are maintaining carnitine homoeostasis (Vaz and Wanders 2002). γ-Butyrobetaine is the last intermediate in the carnitine biosynthesis pathway and is hydroxylated only in the liver by γ-butyrobetaine dioxygenase (EC 1.14.11.1) to yield carnitine (Vaz and Wanders 2002). Nevertheless γ-butyrobetaine and its presursors in the carnitine biosynthesis pathway have also been detected in different tissues like muscle, heart and kidney. Moreover, all the enzymes forming these precursors and γ-butyrobetaine were present in these tissues except γ-butyrobetaine dioxygenase (van Vlies et al. 2006).

Characteristically, in VLCAD deficiency long-chain C14-C18 acyl-CoAs accumulate in mitochondria prior to the β-oxidation block. To leave the mitochondria, they are reconverted into acylcarnitine esters, which can be assayed in blood. As a result of an increased production of acylcarnitines, blood free carnitine concentrations may decrease (Spiekerkoetter et al. 2004). Especially physical exercise results in decreased free carnitine concentrations in skeletal muscles (Spiekerkoetter et al. 2005). However, earlier studies have shown that blood and tissue concentrations of free carnitine do not always correlate (Spiekerkoetter et al. 2005). It has been widely discussed whether supplementation of exogenous carnitine is advisable to recover intracellular carnitine concentrations (Spiekerkoetter et al. 2000; Touma et al. 2001). In contrast, an increased supply of carnitine could result in a further increase of

long-chain acylcarnitines, compounds associated with possibly lethal heart rhythm disturbance (Bonnet et al. 1999).

The VLCAD knock-out (KO) mouse is viable and demonstrates no VLCAD-specific clinical phenotype under resting conditions in the first 6 months of life besides an increased fat storage in tissues (Exil et al. 2006). Physical exercise or fasting however, may induce similar clinical phenotypes to humans (Cox et al. 2001). VLCAD KO mice present with stress-induced hypoglycemia, skeletal myopathy and cold intolerance associated with elevated C14-C18 acylcarnitines (Spiekerkoetter et al. 2005). Overall, the VLCAD KO mouse is an excellent model to study human VLCAD deficiency.

In the present study we analyzed the effects of physical exercise, regeneration after physical exercise and the effect of carnitine supplementation on carnitine and acylcarnitine homeostasis in the VLCAD KO mouse. In addition, we analyzed γ-butyrobetaine, a carnitine biosynthesis metabolite, in tissues to define the importance of carnitine supplementation versus endogenous biosynthesis. In order to examine the effects of C16-CoA and C16-carnitine, respectively, on cell proliferation and viability, HepG2 cells were incubated with these metabolites.

Methods

Concentrations of γ-butyrobetaine, carnitine and acylcarnitines in blood and tissues were measured in VLCAD$^{-/-}$ mice and wild-type littermates under well fed, resting conditions. Each group consisted of five mice aged 10-12 weeks. The mean body weight of the 10-12 week-old animals was 24.4 g ± 0.6. In order to determine carnitine and acylcarnitines in liver, skeletal muscle and blood in response to exercise and after 24 h of regeneration, mice from both genotypes (n=5) were subjected to treadmill exercise. A second group of mice from both genotypes (n=5) received oral carnitine supplementation for five weeks after weaning. Also carnitine supplemented mice were investigated under resting conditions, after exercise and after regeneration.

The mice were sacrificed immediately or 24 h after the stress situation was terminated. Liver and skeletal muscle were removed and frozen immediately in liquid nitrogen. Blood samples were collected by heart puncture and dried on a filter paper card.

Generation and genotyping of VLCAD-deficient mice

VLCAD KO mice were generated as described (Papaioannou VE and Johnson RS 1993; Spiekerkoetter et al. 2005). Genotypes were determined by duplicate PCR analyses (Exil et al. 2003).

Exercise on a treadmill

Five VLCAD KO and five wild-type mice were subjected to exercise on a treadmill equipped with an electric shock grid (10 mAmp, frequency of 10 Hz). Mice had to run at a moderate speed of 16 m*min^{-1} for 60 min. Some VLCAD KO mice were exhausted after 45 min. Exhaustion was defined as resting more than 15 s*min^{-1} on the electric shock grid or as falling back on the electric shock grid more than 15 times*min^{-1} (Spiekerkoetter et al. 2004).

Carnitine supplementation

VLCAD KO and wild-type mice received oral carnitine supplementation in an approximate dose of 200 mg*kg^{-1}*day^{-1} dissolved in drinking water (L-Carn®-drinking solution, 100 mg*ml^{-1}, sigma-tau) for five weeks. The approximate volume of drinking water was calculated at 5 ml. The high dose of 200 mg*kg^{-1}*day^{-1} was chosen, after supplementation of carnitine in a dose of 100 mg*kg^{-1}*day^{-1}, in accordance to clinical use, did not result in increased free carnitine concentrations in blood and tissues of VLCAD KO mice in previous experiments. Metabolites were measured under resting, exercised and regenerated conditions.

Analysis of carnitine and acylcarnitines

In blood, carnitine and acylcarnitines were extracted with methanol from DBS (equivalent to 25 µl of blood) and analyzed as their butyl esters using ESI-MS/MS as previously described (Chace et al. 2001; Vreken et al. 1999). Free carnitine (C0) and all even-chain C14-C18 acylcarnitines (saturated and unsaturated) were measured.

In tissues, analysis of γ-butyrobetaine, carnitine and acylcarnitines was performed according to van Vlies et al. (van Vlies et al. 2005). In brief, liver (~ 60 mg) and skeletal muscle (~50 mg) pieces were lyophilized for 12 h including internal standards (16.25 nmol [$^2H_3$]carnitine, for carnitine and γ-butyrobetaine concentrations; 0.05 nmol [$^2H_3$]C_{16}-acylcarnitine, for C14-C18-acylcarnitines). The lyophilized tissues were powderized and dissolved in 1 ml of 80% acetonitrile. After homogenization and centrifugation the supernatant was dried. γ-butyrobetaine, carnitine and acylcarnitines were analyzed by ESI-MS/MS as their butyl esters and resuspended in 100 µl ACN/H_2O (50/50; v/v).

Cell proliferation and cell viability

3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays were used as measure for cell proliferation and cell viability to investigate cytotoxicity of 20 to 120 and 100 to 500 µM of palmitoyl-carnitine and palmitoyl-CoA, respectively, on HepG2 cells by quantifying mitochondrial dehydrogenase activity. HepG2 cells, in the log phase of growth, were plated in 96-well plates at 100.000 cells per well and after 4 h, palmitoyl-carnitine and palmitoyl-CoA were added. Care was taken to neutralize possible inhibitory pH effects of the substances. After 48 hours of incubation at 37° C and 5% CO_2, MTT was added to a final concentration of 250 $\mu g*mL^{-1}$ and incubated for 4 h. Cell lysis and formazan solubilization were achieved by addition of 100 µL 10% (w/v) sodium dodecyl sulphate. The formazan production was determined spectrophotometrically at 550 nm, with 670 nm as reference. The inhibitory effect of palmitoyl-carnitine and palmitoyl-CoA was objectified by calculating the half maximal inhibitory concentration (IC_{50}) by non-linear curve-fitting of a 5-parameter logistic function to the experimental data.

Statistical analyses

Data are presented as means ± SEM with *n* denoting the number of animals tested. Analyses for the significance of differences were performed using Student's *t* tests for paired and unpaired data ($p < 0.05$).

Results

Free carnitine in skeletal muscle. Free carnitine concentrations were determined in skeletal muscle under resting conditions. In wild-type and VLCAD KO mice free carnitine concentrations were comparable (Fig. 1a). We further studied the effect of exercise on free carnitine concentrations. Free carnitine decreased in muscle from wild-type mice and VLCAD KO mice. Importantly, this decrease in carnitine was significantly greater in VLCAD KO mice, compared to wild-type mice, leading to lower levels. After 24 h of regeneration, carnitine concentrations increased to initial levels, also in VLCAD KO mice. Moreover, carnitine supplementation of wild-type and VLCAD KO mice did not increase free carnitine in skeletal muscle under resting conditions, and did not prevent low carnitine concentrations after exercise in VLCAD KO mice (Fig. 1a).

Free carnitine in liver. Concentrations were significantly increased in exercised VLCAD KO mice compared to resting conditions (Fig. 1b). With carnitine supplementation, exercised mice also presented with significantly higher free carnitine levels as compared to

resting conditions, however, concentrations were lower than without carnitine supplementation. After regeneration, free carnitine concentrations in liver tissue significantly decreased with carnitine supplementation but remained high without carnitine supplementation (Fig. 1b).

Fig. 1a) muscle

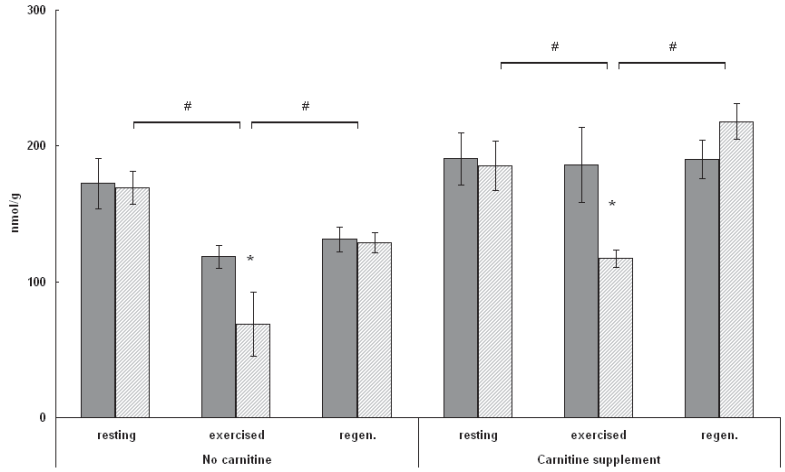


Fig. 1b) liver

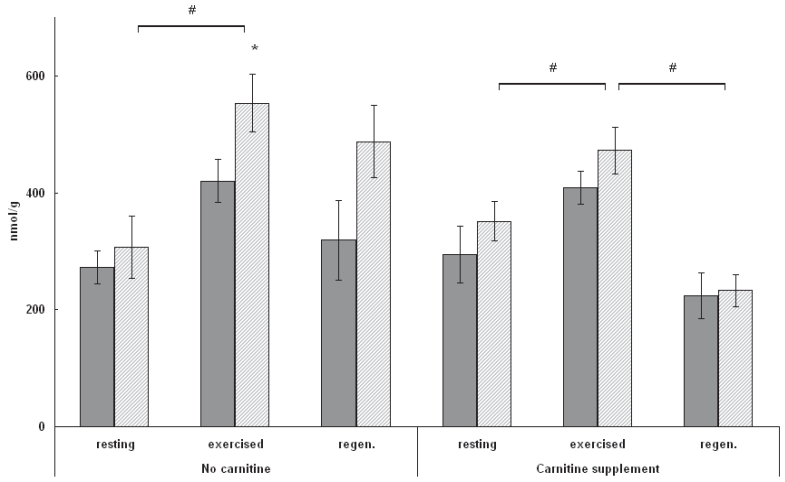


Fig. 1c) blood

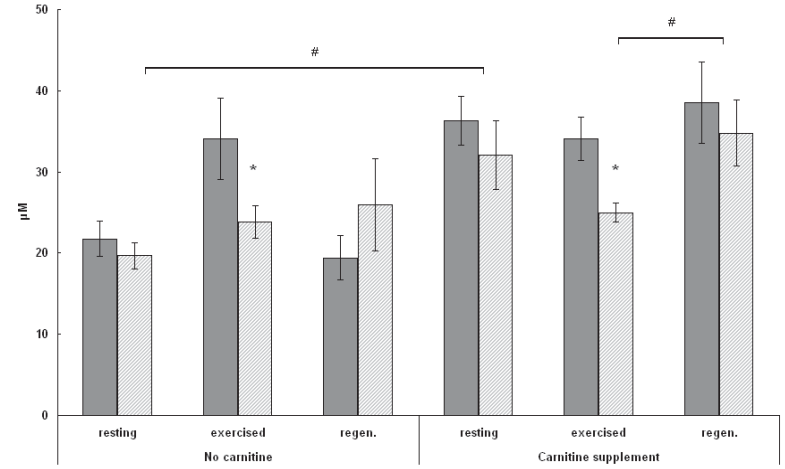


Figure 1: Free carnitine concentrations in blood and tissues

Mean free carnitine concentrations in muscle (a; nmol*g^{-1} wet weight), liver (b; nmol*g^{-1} wet weight) and blood (c; µmol*L^{-1}) under resting, exercised and regenerated conditions are shown in VLCAD KO and wild-type mice and carnitine supplemented VLCAD KO and wild-type mice. Grey bars and striped bars represent wild-type and VLCAD KO mice, respectively.

Values are means ± s.e.m. for five mice, where each tissue was analysed in duplicate. * $P < 0.05$ indicating significant differences between wild-type and VLCAD KO mice. # $P < 0.05$ indicating significant differences between VLCAD KO mice in different groups.

Free carnitine in blood. Carnitine supplementation resulted in higher carnitine levels under resting conditions (Fig. 1c). In wild-type mice without carnitine supplementation free carnitine increased significantly after excerise; in VLCAD KO mice no significant changes were observed. After regeneration free carnitine decreased again in wild-type mice, with respect to the exercised group. In VLCAD KO mice, regeneration had no influence on blood carnitine. After exercise, also carnitine-supplemented VLCAD KO mice presented with significantly lower free carnitine, however, exercise had no effect on blood carnitine levels in wild-type mice. After regeneration, blood carnitine concentrations returned to initial levels in VLCAD KO mice (Fig. 1c).

Long-chain acylcarnitines. In skeletal muscle tissue, after physical exercise, C14-C18 acylcarnitine concentrations significantly increased in VLCAD KO mice and decreased again after 24 h of regeneration (Fig. 2a). Carnitine-supplemented VLCAD KO mice displayed significantly higher concentrations of acylcarnitines in skeletal muscle tissue under resting conditions. Exercise resulted in a further increase of long-chain acylcarnitines in carnitine supplemented mice. Concentrations were four-fold higher than in resting mice without carnitine supplementation. After 24 h of regeneration, acylcarnitine concentrations decreased to lower levels than found under resting conditions before exercise (Fig. 2a).

In liver, acylcarnitine concentrations were elevated after exercise and decreased again in regenerated mice to levels observed at rest. Interestingly, carnitine supplementation resulted in three-fold higher acylcarnitine concentrations in both wild-type and VLCAD KO resting mice, as compared to mice without carnitine supplementation. In both, wild-type and VLCAD KO mice, with supplementation of carnitine, there was no change in acylcarnitine concentrations under resting, exercised or regenerated conditions (Fig. 2b).

Concentrations of C14-C18 acylcarnitines in blood of wild-type mice remained unchanged after exercise and regeneration. VLCAD KO mice displayed higher acylcarnitine levels after exercise. Carnitine supplementation resulted in increased acylcarnitines in VLCAD KO mice compared to wild-type mice (Fig. 2c).

γ-Butyrobetaine. Gamma-butyrobetaine concentrations were measured as precursor of carnitine in endogenous carnitine biosynthesis. In skeletal muscle, γ-butyrobetaine concentrations significantly decreased in VLCAD KO mice after exercise. Carnitine supplemented mice displayed, overall, much higher concentrations of γ-butyrobetaine in skeletal muscle. However, γ-butyrobetaine levels were significantly lower in muscle from VLCAD KO mice as compared to wild-type littermates (Fig. 3a).

Fig. 2a) muscle

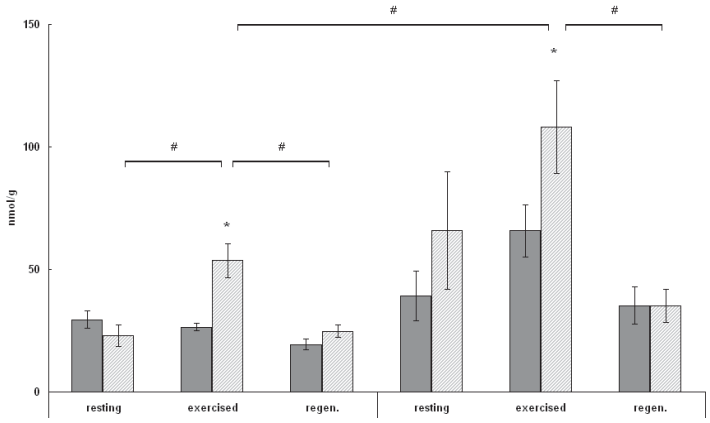


Fig. 2b) liver

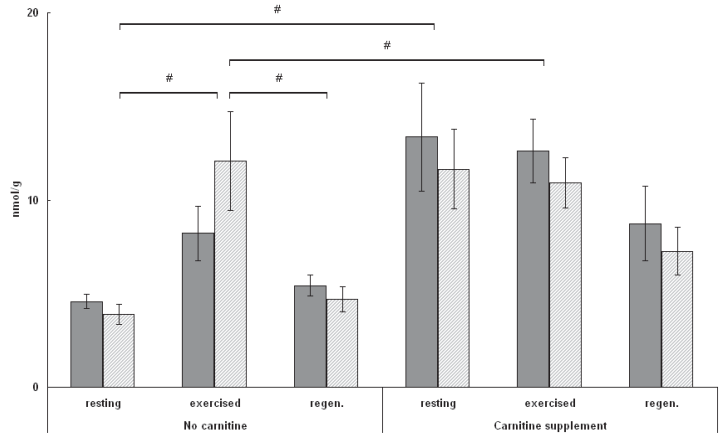


Fig. 2c) blood

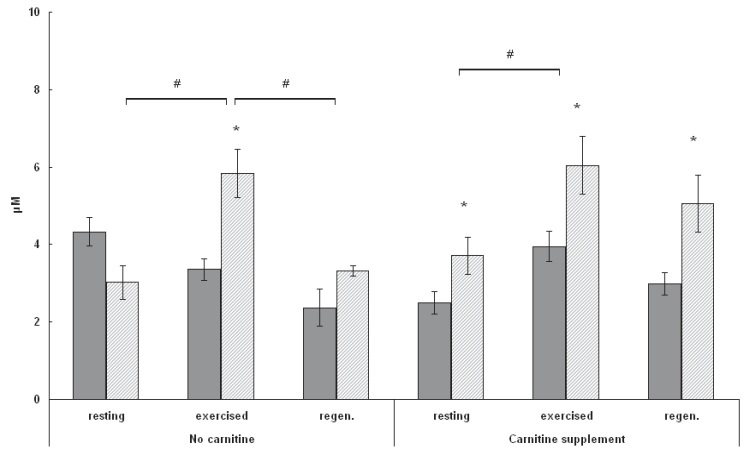


Figure 2: Acylcarnitine concentrations in blood and tissues
Mean acylcarnitine concentrations in muscle (a; nmol g^{-1} wet weight), liver (b; nmol*g^{-1} wet weight) and blood (c; µmol*L^{-1}) under resting, exercised and regenerated conditions are shown in VLCAD KO and wild-type mice and carnitine supplemented VLCAD KO and wild-type mice. Grey bars and striped bars represent wild-type and VLCAD KO mice, respectively.
Values are means ± S.E.M. for five mice, where each tissue was analysed in duplicate. * $P < 0.05$ indicating significant differences between wild-type and VLCAD KO mice. # $P < 0.05$ indicating significant differences between VLCAD KO mice in different groups.

In liver, γ-butyrobetaine concentrations remained in VLCAD KO mice at the same level after exercise and after regeneration, although wild-type mice displayed significantly increased γ-butyrobetaine concentrations after exercise. Carnitine supplementation also resulted in significantly higher liver γ-butyrobetaine concentrations. Importantly, γ-butyrobetaine was significantly lower in VLCAD KO mice as compared to wild-type mice after 24 h of regeneration (Fig. 3b).

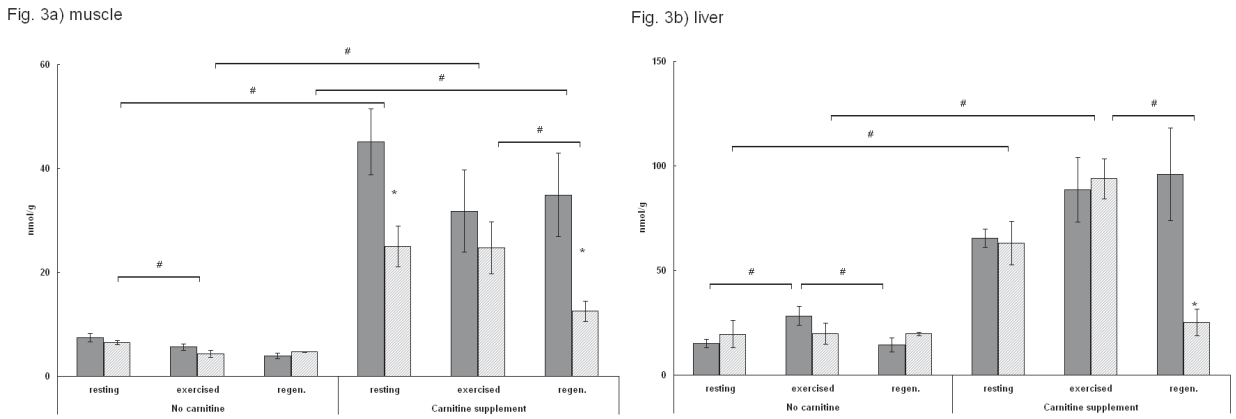


Figure 3: γ-butyrobetaine concentrations in muscle and liver tissue
Mean γ-butyrobetaine concentrations in muscle (a; nmol*g^{-1} wet weight) and liver (b; nmol*g^{-1} wet weight) under resting, exercised and regenerated conditions are shown in VLCAD KO and wild-type mice and carnitine supplemented VLCAD KO and wild-type mice. Grey bars and striped bars represent wild-type and VLCAD KO mice, respectively. Values are means ± S.E.M. for five mice, where each tissue was analysed in duplicate. * $P < 0.05$ indicating significant differences between wild-type and VLCAD KO mice. # $P < 0.05$ indicating significant differences between VLCAD KO mice in different groups.

Cell proliferation and cell viability. Proliferation and viability of HepG2 cells is significantly affected after incubation with C16:0-carnitine in the MTT-assay. The IC_{50} for cells with C16:0-CoA was 337 µM. Cells with C16:0-carnitine had an IC_{50} of 76 µM (Fig. 4). So the cytotoxicity of C16:0-carnitine is four-fold higher than the cytotoxicity of C16:0-CoA.

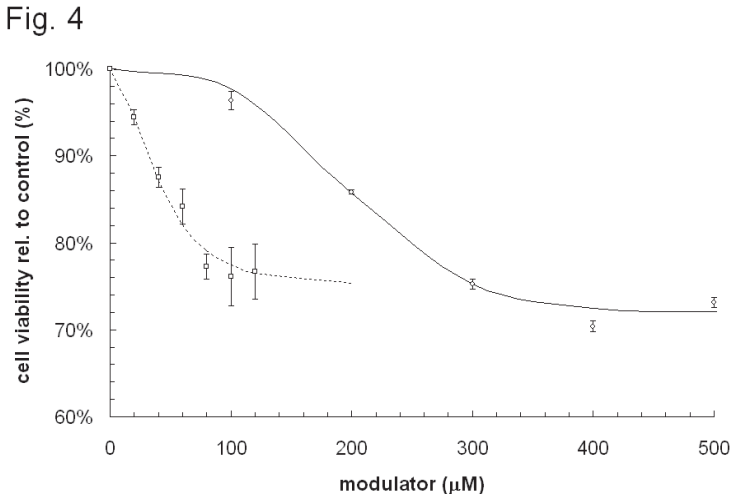


Figure 4: Cell proliferation and cell viability
Response of HepG2 cells to palmitoyl-carnitine and palmitoyl-CoA revealed by MTT assay. HepG2 cells were plated and incubated 4 hours later with C16:0-CoA (squares) or C16:0-carnitine (circles), respectively, for 48 h. Curves represent non-linear curve-fitting of a 5-parameter logistic function to the experimental data.

Discussion

In the present study we demonstrated the effect of carnitine supplementation on exercise-induced changes in carnitine and acylcarnitine concentrations in skeletal muscle, liver and blood from wild-type and VLCAD KO mice. The dose of supplemented carnitine was chosen double compared to usual clinical use as previous experiments in VLCAD KO mice showed that low carnitine levels after exercise were not prevented with the usual dose of 100 mg*kg^{-1}*day^{-1} (Liebig et al. 2006). In addition to the metabolic steps involved in the consumption of carnitine we also analyzed γ-butyrobetaine in both murine skeletal muscle and liver in order to gain more insight into the intrinsic pathways of carnitine biosynthesis.

Carnitine supplementation and acylcarnitine concentrations

Our current study in the murine model of VLCAD deficiency demonstrates for the first time a significant rise in acylcarnitines observed in skeletal muscle when mice are supplemented with carnitine (Liebig et al. 2006). With exercise and an increase in acyl-CoA, the production of acylcarnitines after carnitine supplementation is even greater compared to animals without carnitine supplementation. This has previously been assumed, but it has never been shown at tissue level. Indication for carnitine supplementation in disorders with secondary carnitine deficiency such as VLCAD deficiency has been 1) increased urinary elimination of accumulating acyl-CoAs as acylcarnitines and 2) provision of sufficient intramitochondrial CoA for other metabolic pathways. The significant production of acyl-carnitines after carnitine supplementation, was observed irrespective of the studied genotype and questions the beneficial role of carnitine supplementation in patients, although being based on a mouse-model. Of note, it is well documented that both acylcarnitines as well as acyl-CoAs are potentially toxic for the cells as they may be inhibiting other important pathways in the cells (Adams et al. 1979; Ciapaite et al. 2006). Due to the fact that the increase of acylcarnitines induced by carnitine supplementation is more pronounced in VLCAD KO mouse muscle, the overall result of exercise in combination with carnitine supplementation is a near five-fold increase in acylcarnitine levels in VLCAD KO mouse muscle as compared to muscle from non-supplemented wild-type mice under resting conditions.

Similar to muscle from carnitine supplemented mice, liver acylcarnitine levels are increased upon carnitine supplementation, albeit not as much as in muscle. Since liver oxidative capacity is far lower than in skeletal muscle, concentrations of acylcarnitines in

liver tissue are overall lower than in skeletal muscle. Our studies on the cytotoxic effects of palmitoylcarnitine and palmitoyl-CoA in proliferating hepatic cells revealed approximately four-fold higher cytotoxicity of palmitoylcarnitine, illustrating the negative effect of acylcarnitine accumulation in liver cells (Fig. 4).

Free carnitine and acylcarnitine dynamics in blood and skeletal muscle

As expected, carnitine supplementation results in a near two-fold increase in blood carnitine concentration in resting wild-type and VLCAD KO mice. After exercise free carnitine in blood is significantly lower in VLCAD KO mice as compared to wild-type mice, irrespective of carnitine supplementation. These low blood concentrations reflect the decreased levels in VLCAD KO mouse muscle and suggest impaired carnitine handling under exercised conditions with and without supplementation of carnitine. Supplemented carnitine, therefore, fails to prevent the significant drop in muscle free carnitine under exercised conditions. But even without supplemented carnitine, free carnitine levels replenish again within 24 h of regeneration. Furthermore, carnitine supplementation does not prevent increased blood acylcarnitine levels after exercise. Acylcarnitines are high in blood as soon as there are increased concentrations in any organ, because they are thought to leave the cells and go into the circulation to finally be excreted via the urine from the body (Bennett et al. 2000; Rinaldo 2001). Overall, based on our current findings, using the murine model of VLCAD deficiency, carnitine supplementation does not prevent low carnitine concentrations in muscle after exercise and moreover, supplementation actually results in a sharp rise of acylcarnitines in skeletal muscle.

Liver carnitine biosynthesis is no longer coupled to muscle carnitine demand during carnitine supplementation

As a marker for carnitine biosynthesis we studied γ-butyrobetaine levels in murine liver and skeletal muscle and found that carnitine supplementation greatly influences the dynamics of this precursor. First we observed the profound effect of exercise stress on γ-butyrobetaine in skeletal muscle in VLCAD KO mice, with considerably lower γ-butyrobetaine concentrations. As skeletal muscle does not possess the full machinery to biosynthesize carnitine, muscle tissue lacks γ-butyrobetaine dioxygenase (van Vlies et al. 2006), we assume that the observed decrease in γ-butyrobetaine after exercise in VLCAD KO mice represents transport of γ-butyrobetaine out of the muscle cell in order to supply precursor for the biosynthesis of carnitine in liver. Also Vaz and Wanders (Vaz and Wanders 2002) postulate

that γ-butyrobetaine is produced in different kinds of tissues and then excreted into the circulation to be transported to a tissue that contains γ-butyrobetaine dioxygenase. Overall, after carnitine supplementation γ-butyrobetaine concentrations are significantly higher irrespective of the genotype suggesting decreased demand of carnitine biosynthesis. However, VLCAD KO mice presented with significantly lower γ-butyrobetaine concentrations in muscle than wild-type mice and after 24 h of regeneration γ-butyrobetaine was significantly decreased as compared to resting conditions. These findings might implicate that in all non-supplemented animals and in supplemented VLCAD KO mice there is still a higher demand for carnitine biosynthesis, especially during exercise stress because liver carnitine production is augmented (Fig. 2b). The observed increase in liver carnitine concentration during exercise, with and without carnitine supplementation, is indicative of γ-butyrobetaine being converted into carnitine by γ-butyrobetaine dioxygenase rather than liver γ-butyrobetaine production being reduced. This would be in-line with the observed decrease in γ-butyrobetaine in liver after exercise or within 24 h of regeneration. Importantly, after 24 h of regeneration, liver carnitine levels remain high in non-supplemented VLCAD KO mice, indicating an increased and sustained demand for carnitine in this mouse phenotype during the recovery phase. In VLCAD KO mice receiving oral supplementation of carnitine, liver carnitine returns to basic levels after 24 h of regeneration reflecting that high blood carnitine in carnitine supplemented VLCAD KO animals suppresses carnitine biosynthesis in liver, at least to some degree. Whether the gene for γ-butyrobetaine dioxygenase is upregulated in VLCAD KO mice or upregulated after regular exercise in response to the secondary carnitine deficiency is currently under investigation.

Blood carnitine and acylcarnitine profiling does not fully mirror cellular carnitine metabolism

Within the framework of clinical diagnostics, acylcarnitine profiling of DBS represents a powerful tool for clinicians to gain insight into fatty acid metabolism. However, blood acylcarnitine profiles can merely represent an overall response of all organs involved in carnitine metabolism. The consequence of this is that blood acylcarnitines do not necessarily reflect concentration changes taking place in individual organ systems (Spiekerkoetter et al. 2005), thus even a moderate increase in blood acylcarnitines should be treated with caution. For example, we observed increased acylcarnitine levels in skeletal muscle after oral supplementation of carnitine whereas blood acylcarnitine levels remained nearly unchanged.

Only under stressed conditions blood acylcarnitines actually reflect acylcarnitine dynamics in skeletal muscle, but not at rest.

In conclusion, in the murine model of VLCAD deficiency we observed a significant increase in acylcarnitine production after oral supplementation of carnitine in skeletal muscle, especially after exercise. However, carnitine supplementation does not prevent decreased free carnitine in muscle after exercise. Furthermore, exercise triggers carnitine biosynthesis with replenishment of low free carnitine pools in muscle within 24 hours. Therefore, if we extrapolate our findings obtained after one hour of moderate stress in this mouse model, it may well be that carnitine supplementation is not at all beneficial for affected patients with VLCAD deficiency. More importantly, we rather have to consider that carnitine supplementation may actually be a risk factor because carnitine supplementation results in significant accumulation of toxic acylcarnitines in mice tissues.

Acknowledgements

The study was financially supported by grants from the Deutsche Forschungsgemeinschaft (DFG; SP1125/1-1) and the Medical Faculty of Heinrich-Heine-University, Duesseldorf, Germany. We thank Dr. M. Laryea and G. Schmitz for technical assistance.

References

1. Adams RJ, Cohen DW, Gupte S, Johnson JD, Wallick ET, Wang T, Schwartz A (1979) In vitro effects of palmitylcarnitine on cardiac plasma membrane Na,K-ATPase, and sarcoplasmic reticulum Ca2+-ATPase and Ca2+ transport. J Biol Chem 254:12404-12410

2. Bennett MJ, Rinaldo P, Strauss AW (2000) Inborn errors of mitochondrial fatty acid oxidation. Crit Rev Clin Lab Sci 37:1-44

3. Bonnet D, Martin D, Pascale DL, Villain E, Jouvet P, Rabier D, Brivet M, Saudubray JM (1999) Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children. Circulation 100:2248-2253

4. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, Hofman LF, Naylor EW (2001) Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. Clin Chem 47:1166-1182

5. Ciapaite J, Bakker SJ, Diamant M, van EG, Heine RJ, Westerhoff HV, Krab K (2006) Metabolic control of mitochondrial properties by adenine nucleotide translocator determines palmitoyl-CoA effects. Implications for a mechanism linking obesity and type 2 diabetes. FEBS J 273:5288-5302

6. Cox KB, Hamm DA, Millington DS, Matern D, Vockley J, Rinaldo P, Pinkert CA, Rhead WJ, Lindsey JR, Wood PA (2001) Gestational, pathologic and biochemical differences between very long-chain acyl-CoA dehydrogenase deficiency and long-chain acyl-CoA dehydrogenase deficiency in the mouse. Hum Mol Genet 10:2069-2077

7. Exil VJ, Gardner CD, Rottman JN, Sims H, Bartelds B, Khuchua Z, Sindhal R, Ni G, Strauss AW (2006) Abnormal mitochondrial bioenergetics and heart rate dysfunction in mice lacking very-long-chain acyl-CoA dehydrogenase. Am J Physiol Heart Circ Physiol 290:H1289-H1297

8. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

9. Gregersen N, Bross P, Andresen BS (2004) Genetic defects in fatty acid beta-oxidation and acyl-CoA dehydrogenases. Molecular pathogenesis and genotype-phenotype relationships. Eur J Biochem 271:470-482

10. Liebig M, Gyenes M, Brauers G, Ruiter JP, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Carnitine supplementation induces long-chain acylcarnitine production--studies in the VLCAD-deficient mouse. J Inherit Metab Dis 29:343-344

11. Papaioannou VE, Johnson RS (1993) Gene targeting: a practical approach. Oxford University Press, Oxford107-146

12. Rinaldo P (2001) Fatty acid transport and mitochondrial oxidation disorders. Semin Liver Dis 21:489-500

13. Spiekerkoetter U, Schwahn B, Korall H, Trefz FK, Andresen BS, Wendel U (2000) Very-long-chain acyl-coenzyme A dehydrogenase (VLCAD) deficiency: monitoring of treatment by carnitine/acylcarnitine analysis in blood spots. Acta Paediatr 89:492-495

14. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U (2003) MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr 143:335-342

15. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

16. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van VN, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

17. Touma EH, Rashed MS, Vianey-Saban C, Sakr A, Divry P, Gregersen N, Andresen BS (2001) A severe genotype with favourable outcome in very long chain acyl-CoA dehydrogenase deficiency. Arch Dis Child 84:58-60

18. van Vlies N, Wanders RJ, Vaz FM (2006) Measurement of carnitine biosynthesis enzyme activities by tandem mass spectrometry: differences between the mouse and the rat. Anal Biochem 354:132-139

19. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

20. Vaz FM, Wanders RJ (2002) Carnitine biosynthesis in mammals. Biochem J 361:417-429

21. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH (1999) Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. J Inherit Metab Dis 22:302-306

Chapter 3

ESI-MS/MS measurement of free carnitine and its precursor γ-butyrobetaine in plasma and dried blood spots from patients with organic acidurias and fatty acid oxidation disorders

Sonja Primassin and Ute Spiekerkoetter

Molecular Genetics and Metabolism, 2010 Jun; accepted for publication
(DOI: 10.1016/j.ymgme.2010.06.012)

Abstract

Background. In patients with fatty acid oxidation disorders (FAODs) and organic acidurias (OAs) "secondary carnitine deficiency" occurs. In OAs carnitine supplementation is widely performed and dose is often adjusted to blood free carnitine levels. Dried blood spots (DBS) are mostly used to measure carnitine status, however measurements in plasma are discussed to be more accurate. The concentration and the predictive value of *the carnitine precursor γ-butyrobetaine in blood during carnitine deficiency are unknown.*

Methods. Free carnitine and γ-butyrobetaine were quantified by tandem mass spectrometry in plasma and DBS from supplemented patients with OAs (n = 18) and unsupplemented patients with FAODs (n = 66) and were compared with healthy controls (n = 50).

Results. Carnitine concentrations in plasma were significantly higher than in DBS. In contrast, γ-butyrobetaine concentrations in plasma were significantly lower than in DBS. Supplemented patients had high free carnitine concentrations in combination with high γ-butyrobetaine concentrations. Unsupplemented carnitine palmitoyltransferase I-deficient patients had exceptionally high free carnitine concentrations without elevated γ-butyrobetaine, however, carnitine in plasma was much lower than in DBS. In patients with low carnitine, γ-butyrobetaine in plasma is no evidence of induced carnitine biosynthesis.

Conclusions. Parallel measurements in plasma and DBS demonstrated that numerous patients with low values in DBS had normal values when measured in plasma, suggesting plasma to be the more appropriate medium to use for carnitine status monitoring. In contrast, diagnosis of CPT-I deficiency may be missed when analysis is performed in plasma. Carnitine supplementation presumably inhibits γ-butyrobetaine dioxygenase and results in high γ-butyrobetaine.

Introduction

Inherited metabolic disorders are commonly mass screened using electrospray ionization mass spectrometry (ESI-MS/MS) in many countries worldwide. Several disorders including organic acidurias (OAs) and fatty acid oxidation disorders (FAODs) can be detected using this method (Chace et al. 2003; Schulze et al. 2003; Wilcken et al. 2003). Diagnosis of various FAODs and OAs is possible due to disease-specific acylcarnitine profiles (Vreken et al. 1999). Generally, measurements are performed in dried blood spots (DBS). Analysis is also used to monitor the free carnitine status, especially in patients under L-carnitine supplementation. "Secondary carnitine deficiency" is reported in patients with OAs and FAODs and occurs due to increased production of acylcarnitines and loss of carnitine species via urine and bile (Stanley 1995). Because of low blood concentrations L-carnitine is often considered for therapy. Carnitine is an endogenous compound present in most mammalian tissues and is either ingested with the diet or produced endogenously in liver, kidney or brain (Rebouche and Engel 1980). Supplementation with L-carnitine, either orally or intravenously, has been shown to increase the carnitine plasma concentration (Evans and Fornasini 2003), but it may also increase acylcarnitine concentrations (Primassin et al. 2008). Long-chain fatty acids are bound to carnitine to cross the mitochondrial membranes for subsequent β-oxidation (Hoppel 2003). Carnitine is also involved in the regulation of the cellular pool of free coenzyme A (CoA) by accepting acyl-groups from acyl-CoAs. This buffer effect may be important to neutralize the potentially toxic acyl-groups as acylcarnitines that can be excreted in the urine, in contrast to acyl-CoAs (Hoppel 2003; Roe et al. 1983).

It still remains unclear whether carnitine supplementation helps to prevent secondary carnitine deficiency, especially with respect to tissue levels (Primassin et al. 2008). The usefulness of carnitine blood levels for treatment monitoring also remains questionable. Therefore, we here evaluated DBS and plasma samples from numerous patients to directly compare these two blood compounds with respect to the carnitine status. It would also be of great interest to be able to determine the contribution of endogenous carnitine biosynthesis in blood. However, it remains unknown whether γ-butyrobetaine, the precursor of carnitine, can be used as a marker for carnitine biosynthesis and the extent of carnitine deficiency. In the current study, we investigated concentrations of free carnitine and γ-butyrobetaine in plasma and DBS of patients with FAODs without L-carnitine supplementation and in patients with OAs with L-carnitine supplementation and compared the data with healthy controls.

Subjects and methods

Participants

Plasma and DBS were obtained from subjects with enzymatic or molecular diagnosis of FAODs and OAs. All patient studies were performed anonymously with parental consent. Measurements were part of routine treatment monitoring. OA patients received L-carnitine in a dose of 100 mg/kg body weight. FAOD patients did not receive L-carnitine. Analyses were performed in 39 patients with medium-chain acyl-CoA dehydrogenase (MCAD; EC 1.3.99.3) deficiency (OMIM #201450) at the age of 1-13 years, in 16 patients with very-long-chain acyl-CoA dehydrogenase (VLCAD; EC 1.3.99.3) deficiency (OMIM #201475) at the age of 1-12 years, in eight patients with glutaric aciduria type I (GA-I; OMIM #231670) at the age of 2-10 years, in four patients with methylmalonic aciduria (MMA; OMIM #251000) at the age of 3-18 years, in three patients each with long-chain 3-hydroxyacyl-CoA dehydrogenase (LCHAD; EC 1.1.1.35) deficiency (OMIM #609016) at the age of 2-10 years, multiple acyl-CoA dehydrogenase (MAD) deficiency (OMIM #231680) at the age of 3-6 years, carnitine palmitoyltransferase I (CPT-I; EC 2.3.1.21) deficiency (OMIM #255120) at the age of 2-5 years and isovaleric aciduria (IVA; OMIM #243500) at the age of 2-13 years, two patients with malonic aciduria (MA; OMIM #248360) at the age of 2-6 years and in one patient each with carnitine palmitoyltransferase II (CPT-II; EC 2.3.1.21) deficiency (OMIM #255110) at the age of 12 years, trifunctional protein (TFP) deficiency (OMIM #609015) at the age of 19 years and propionic aciduria (PA; OMIM #606054) at the age of 6 years. Some patients were measured repeatedly in routine check-up. All participants were well at time of sample collection.

Reagents

The [2H3] carnitine (C0) internal standard was obtained from Dr Herman J. ten Brink (VU Medical Hospital, Amsterdam, Netherlands). LC/MS-grade solvents, methanol and acetonitrile were obtained from Fisher Scientific.

Sample collection and preparation

Blood samples were collected by venous puncture and a small amount was dropped on a Protein Saver™ 903® Card (Whatman GmbH, Dassel, Germany) and dried for 24 h. The remaining blood was centrifuged and the resulting plasma was separated and frozen at -80°C until use. Free carnitine and γ-butyrobetaine were extracted from DBS or from 5 µl plasma with methanol (100 %) containing internal standard (16.25 nmol [$^2H_3$] carnitine). For the extraction and analysis, punches of 3-mm diameter from the DBS specimens were used. After

an incubation time of 20 min, plasma and DBS samples were comparably filtered through 96-well 1.2 µm filter plates (Millipore, Schwalbach, Germany) and then dried at 40°C under air flow. Dried DBS and plasma samples were derivatized with butanol 15 min at 65°C and then dried again at 40°C under air flow. After addition of 100 µl acetonitrile/H_2O (1:1) the DBS and plasma samples were measured by ESI-MS/MS.

Quantitative analysis of carnitine and γ-butyrobetaine using electrospray tandem mass spectrometry

Free carnitine and γ-butyrobetaine were analyzed as their butyl esters using ESI-MS/MS as described previously (Chace et al. 2001; van et al. 2005; Vreken et al. 1999). Control ranges were obtained from plasma of 50 healthy individuals (aged 2-17 years).

Statistical analysis

Data were acquired and analyzed using MassLynx NT v4.0 (Micromass, UK). Data are expressed as the means ± standard error of the mean (SEM). Statistical analysis of differences between two means was assessed by Student's *t* test. Multiple means were compared by a two-way analysis of the variance (ANOVA) with a Bonferroni post hoc test (GraphPad Prism 4·0, San Diego, CA, USA). A probability level of $p<0.05$ was regarded as significant.

Results and Discussion

Carnitine and γ-butyrobetaine concentrations in plasma and dried blood spots

In this study we compared free carnitine concentrations measured in plasma and DBS samples. Johnson (Johnson 1999) already claimed that measurement of free carnitine by ESI-MS/MS in DBS is inaccurate. According to our data free carnitine concentrations measured in plasma samples were generally 3.12 (± 0.13) times higher than free carnitine concentrations measured in DBS (Figure 2A). Borum (Borum 1987) demonstrated that blood free carnitine is partitioned into a plasma carnitine compartment and a red blood cell carnitine compartment. Plasma does not contain the red blood cell compartment, whereas DBS still contain red blood cells. So the different carnitine concentrations found in plasma and DBS samples are likely due to extraction from the different blood compartments. DBS extraction seems to be insufficient due to lower free carnitine concentrations compared with plasma. The normal range for free carnitine in plasma obtained by our 50 healthy individuals was 21-59 µmol/L corresponding with measured ranges shown by Schmidt-Sommerfeld et al. (Schmidt-Sommerfeld et al. 1988). In DBS samples the normal range for free carnitine obtained by our

50 healthy individuals was 12-59 µmol/L. Also the normal range values of 10-70 µmol/L tested by our laboratory from a population of 500 children (unpublished data) was in line with published reference ranges from DBS samples (Cavedon et al. 2005; Sim et al. 2001). The smaller normal range in plasma may be evidence for a more efficient and reproducible extraction of free carnitine from plasma samples. Importantly, plasma free carnitine concentrations were more often within the normal range in patients with inborn errors of metabolism, especially in VLCAD- and MCAD-deficient patients, than in DBS samples (Figure 1A-D). Six VLCAD-deficient patients, 15 MCAD-deficient patients, three LCHAD-deficient patients and one patient with MAD-deficiency presented free carnitine concentrations below the normal limit in DBS samples, but had normal values when measured in plasma. They were, therefore, uncorrectly identified as having "secondary carnitine deficiency". Also patients receiving an L-carnitine supplement often presented free carnitine concentrations below the normal range when measured in DBS samples. In contrast, plasma free carnitine concentrations in patients receiving L-carnitine were often above the normal range as expected (Figure 1E-H). Since free carnitine concentrations in blood are used for treatment monitoring, it is essential to correctly mirror the in vivo carnitine status. However, there are important observations in mouse models that free carnitine in blood is not reflecting carnitine homeostasis in tissues (Primassin et al. 2008).

Whereas free carnitine concentrations were generally higher in blood from patients receiving an L-carnitine supplement, CPT-I-deficient patients, as expected, also presented with significantly elevated free carnitine concentrations in plasma and DBS samples. Interestingly, in this patient group, free carnitine was even higher in DBS samples compared to plasma samples (Figure 2A). We speculate that in this case, there may be a disease dependent free carnitine overload in red blood cells. In healthy individuals 37% of the total carnitine is found in red blood cells (Borum 1987). If most of the carnitine is stored in red blood cells in case of CPT-I deficiency, it will not be extracted and measured in plasma samples. Consequently, measurements in plasma may presumably not correctly identify CPT-I-deficient patients.

In general, γ-butyrobetaine was found in much lower concentrations in plasma compared with DBS samples in all metabolic disorders investigated (Figure 2B) suggesting it is predominantly present in red blood cells. Those patients identified with "secondary carnitine deficiency" in both, plasma and DBS samples (n = 7), did not present significantly different γ-butyrobetaine concentrations than controls (Figure 1).

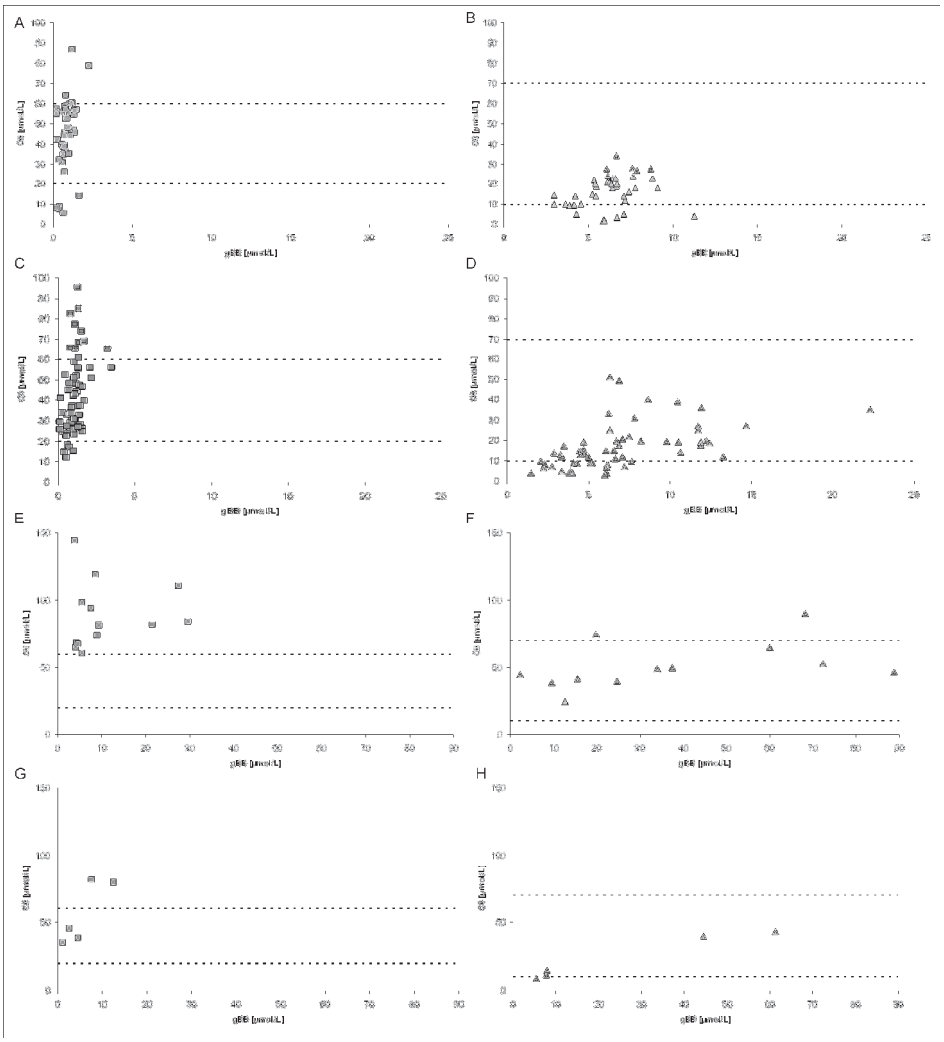


Figure 1: Free carnitine concentrations (C0; µmol/L) in plasma (left) and dried blood spots (right) from patients with VLCAD deficiency (A and B), MCAD deficiency (C and D), GA-I (E and F) and IVA (G and H) as a function of the corresponding γ-butyrobetaine concentrations (gBB; µmol/L) measured in the same samples. Free carnitine reference range is indicated by dotted lines for plasma and dried blood spots.

Correlation between free carnitine and γ-butyrobetaine concentrations in plasma and dried blood spots

In general, carnitine and γ-butyrobetaine did not correlate in plasma or DBS samples (Figure 3). However, γ-butyrobetaine was significantly elevated in patients receiving a carnitine supplement, whereas free carnitine in these patients was in general not significantly elevated compared with unsupplemented individuals (Figure 2 A/B). Consequently, L-carnitine supplementation likely inhibits the last enzyme in carnitine biosynthesis, γ-butyrobetaine dioxygenase, resulting in accumulation of γ-butyrobetaine (Evans et al. 2000; Holme et al. 1982; Vaz and Wanders 2002). It has also been previously demonstrated that γ-

butyrobetaine can be formed by enteric bacteria from L-carnitine in rats and humans (Rebouche and Seim 1998).

In contrast, CPT-I-deficient patients, displayed elevated free carnitine levels but no increased γ-butyrobetaine concentrations in both blood compartments. Hence, high endogenous and high exogenous carnitine may have different effects on the biosynthesis pathway. Another explanation could be that acylcarnitine accumulation in tissues in many defects but not in CPT-I deficiency could trigger carnitine biosynthesis resulting in higher γ-butyrobetaine concentrations in blood (Fingerhut et al. 2001).

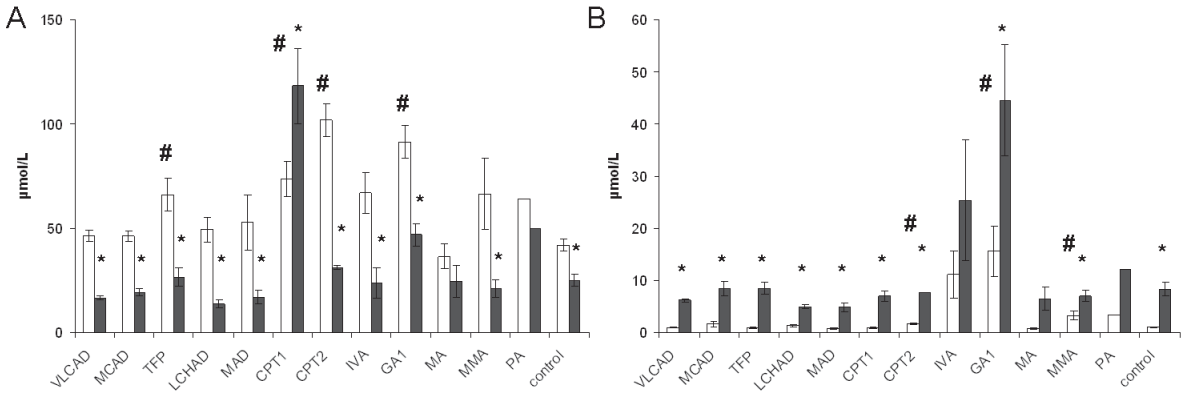


Figure 2: Free carnitine (A) and γ-butyrobetaine (B) concentrations in plasma and dried blood spots. Free carnitine and γ-butyrobetaine concentrations are presented in µmol/L of patients with FAODs without carnitine supplementation, patients with OAs with carnitine supplementation and healthy controls. *White bars* and *black bars* represent plasma and dried blood spot samples, respectively. Values are means ± standard error of the mean (SEM). *$p < 0.05$ indicates significant differences between plasma and dried blood spot samples using Student´s *t* test and #$p < 0.05$ indicates significant differences between patients and healthy controls in plasma samples using two-way analysis of the variance (ANOVA). Samples were analyzed in quadruplicate.

Free carnitine and γ-butyrobetaine in unsupplemented fatty acid oxidation disorders and carnitine-supplemented organic acidurias in plasma

Carnitine deficiency is assumed to be secondary in many inborn errors of metabolism. However, in our cohort of unsupplemented patients with FAODs (VLCAD, MCAD, LCHAD and MAD) free carnitine concentrations were in average comparable to those measured in healthy controls (Figure 2A), suggesting in the majority of patients no secondary carnitine deficiency. Age-related variations as demonstrated by Cavedon et al. (Cavedon et al. 2005), although minor, were eliminated by using a control group of the same age range as reference. In contrast, patients with GA-I under carnitine treatment, but also CPT-I, CPT-II and TFP-

deficient patients showed significantly increased free carnitine concentrations in plasma compared with healthy controls (Figure 2A). Previous studies have demonstrated that endogenous carnitine biosynthesis is induced with increasing carnitine demand such as in VLCAD-deficient mice (Primassin et al. 2008) or with reduced carnitine intake such as in vegetarians (Lombard et al. 1989). Here, we therefore wanted to determine whether γ-butyrobetaine, the precursor of carnitine, could be used as a marker for an increased endogenous carnitine biosynthesis. However, γ-butyrobetaine concentrations were not significantly different in any unsupplemented patients compared with healthy controls (Figure 2B). Moreover, γ-butyrobetaine concentrations were significantly increased in patients with OAs (GA-I, IVA, MMA and PA) under carnitine supplementation compared with healthy controls (Figure 2B). So elevated γ-butyrobetaine concentrations may suggest inhibition of γ-butyrobetaine dioxygenase and therefore do not represent activation of endogenous carnitine biosynthesis.

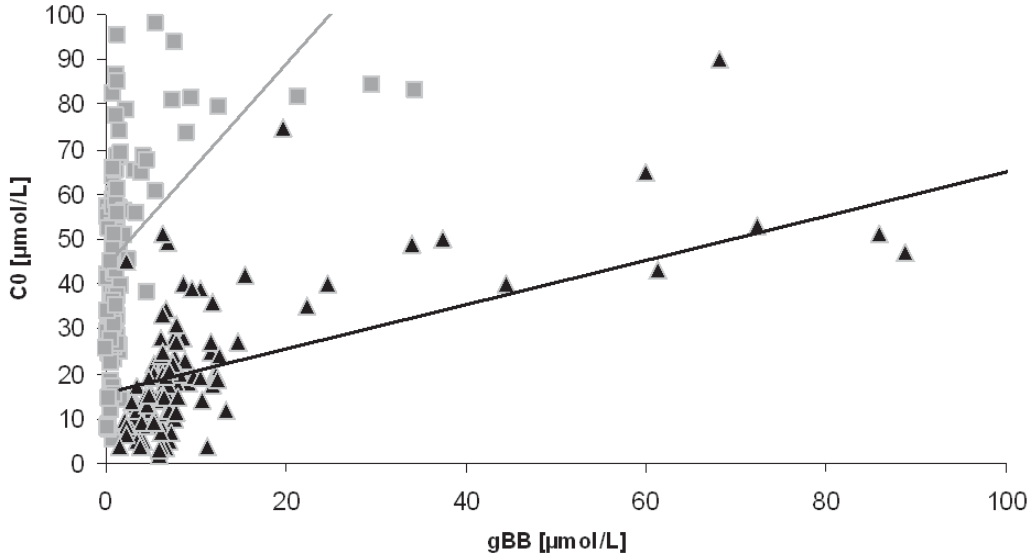


Figure 3: Correlation between free carnitine (C0; µmol/L) concentrations and the corresponding γ-butyrobetaine (gBB; µmol/L) concentrations in plasma (squares) and dried blood (triangles) samples.

Conclusions

Plasma measurements are necessary when monitoring the carnitine status under carnitine supplementation. Surprisingly, the majority of patients with FAO disorders did not present with carnitine deficiency when carnitine is measured in plasma. Carnitine supplementation

may inhibit carnitine biosynthesis as shown by a highly elevated γ-butyrobetaine. Overall, according to our data it is not possible to use γ-butyrobetaine in blood as a marker for the activation status of endogenous carnitine biosynthesis. Interestingly, high endogenous carnitine concentrations, as observed in CPT-I deficiency, are not associated with an elevated γ-butyrobetaine. Whereas free carnitine is significantly elevated in DBS in CPT-I deficiency, concentrations in plasma are much lower and diagnosis may be missed.

Acknowledgments

The study was financially supported by grants from the Deutsche Forschungsgemeinschaft (DFG, SP1125/1-1, SFB 575, SFB 612) and from the Forschungskommission of the Medical Faculty of Heinrich-Heine-University Duesseldorf. We thank Gisela Schmitz and Elke Diekmann for technical assistance and measuring of samples with ESI-MS/MS.

References

1. Borum PR (1987) Plasma carnitine compartment and red blood cell carnitine compartment of healthy adults. Am J Clin Nutr 46:437-441

2. Cavedon CT, Bourdoux P, Mertens K, Van Thi HV, Herremans N, de LC, Goyens P (2005) Age-related variations in acylcarnitine and free carnitine concentrations measured by tandem mass spectrometry. Clin Chem 51:745-752

3. Chace DH, DiPerna JC, Mitchell BL, Sgroi B, Hofman LF, Naylor EW (2001) Electrospray tandem mass spectrometry for analysis of acylcarnitines in dried postmortem blood specimens collected at autopsy from infants with unexplained cause of death. Clin Chem 47:1166-1182

4. Chace DH, Kalas TA, Naylor EW (2003) Use of tandem mass spectrometry for multianalyte screening of dried blood specimens from newborns. Clin Chem 49:1797-1817

5. Evans AM, Faull R, Fornasini G, Lemanowicz EF, Longo A, Pace S, Nation RL (2000) Pharmacokinetics of L-carnitine in patients with end-stage renal disease undergoing long-term hemodialysis. Clin Pharmacol Ther 68:238-249

6. Evans AM, Fornasini G (2003) Pharmacokinetics of L-carnitine. Clin Pharmacokinet 42:941-967

7. Fingerhut R, Roschinger W, Muntau AC, Dame T, Kreischer J, Arnecke R, Superti-Furga A, Troxler H, Liebl B, Olgemoller B, Roscher AA (2001) Hepatic carnitine

palmitoyltransferase I deficiency: acylcarnitine profiles in blood spots are highly specific. Clin Chem 47:1763-1768

8. Holme E, Linstedt S, Nordin I (1982) Uncoupling in the gamma-butyrobetaine hydroxylase reaction by D- and L-carnitine. Biochem Biophys Res Commun 107:518-524

9. Hoppel C (2003) The role of carnitine in normal and altered fatty acid metabolism. Am J Kidney Dis 41:S4-12

10. Johnson DW (1999) Inaccurate measurement of free carnitine by the electrospray tandem mass spectrometry screening method for blood spots. J Inherit Metab Dis 22:201-202

11. Lombard KA, Olson AL, Nelson SE, Rebouche CJ (1989) Carnitine status of lactoovovegetarians and strict vegetarian adults and children. Am J Clin Nutr 50:301-306

12. Primassin S, Ter VF, Mayatepek E, Spiekerkoetter U (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. Pediatr Res 63:632-637

13. Rebouche CJ, Engel AG (1980) Tissue distribution of carnitine biosynthetic enzymes in man. Biochim Biophys Acta 630:22-29

14. Rebouche CJ, Seim H (1998) Carnitine metabolism and its regulation in microorganisms and mammals. Annu Rev Nutr 18:39-61

15. Roe CR, Hoppel CL, Stacey TE, Chalmers RA, Tracey BM, Millington DS (1983) Metabolic response to carnitine in methylmalonic aciduria. An effective strategy for elimination of propionyl groups. Arch Dis Child 58:916-920

16. Schmidt-Sommerfeld E, Werner D, Penn D (1988) Carnitine plasma concentrations in 353 metabolically healthy children. Eur J Pediatr 147:356-360

17. Schulze A, Lindner M, Kohlmuller D, Olgemoller K, Mayatepek E, Hoffmann GF (2003) Expanded newborn screening for inborn errors of metabolism by electrospray ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics 111:1399-1406

18. Sim KG, Wiley V, Carpenter K, Wilcken B (2001) Carnitine palmitoyltransferase I deficiency in neonate identified by dried blood spot free carnitine and acylcarnitine profile. J Inherit Metab Dis 24:51-59

19. Stanley CA (1995) Carnitine disorders. Adv Pediatr 42:209-242

20. van VN, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

21. Vaz FM, Wanders RJ (2002) Carnitine biosynthesis in mammals. Biochem J 361:417-429

22. Vreken P, van Lint AE, Bootsma AH, Overmars H, Wanders RJ, van Gennip AH (1999) Quantitative plasma acylcarnitine analysis using electrospray tandem mass spectrometry for the diagnosis of organic acidaemias and fatty acid oxidation defects. J Inherit Metab Dis 22:302-306

23. Wilcken B, Wiley V, Hammond J, Carpenter K (2003) Screening newborns for inborn errors of metabolism by tandem mass spectrometry. N Engl J Med 348:2304-2312

Chapter 4

A novel tandem mass spectrometry method for rapid confirmation of medium- and very long-chain acyl-CoA dehydrogenase deficiency in newborns

F. ter Veld, M. Mueller, S. Kramer, U. Haussmann, D. Herebian, E. Mayatepek, M. Laryea, S. Primassin and U. Spiekerkoetter

PloS One. 2009:4(7): e6449

Abstract

Background: Newborn screening for medium- and very long-chain acyl-CoA dehydrogenase (MCAD and VLCAD, respectively) deficiency, using acylcarnitine profiling with tandem mass spectrometry, has increased the number of patients with fatty acid oxidation disorders due to the identification of additional milder, and so far silent, phenotypes. However, especially for VLCAD deficiency, the acylcarnitine profile can not constitute the sole parameter in order to reliably confirm disease. Therefore, we developed a new liquid chromatography tandem mass spectrometry (LC-MS/MS) method to rapidly determine both MCAD- and/or VLCAD-activity in human lymphocytes in order to confirm diagnosis.

Methodology: LC-MS/MS was used to measure MCAD- or VLCAD-catalyzed production of enoyl-CoA and hydroxyacyl-CoA, in human lymphocytes.

Principal Findings: VLCAD activity in controls was 6.95 ± 0.42 mU/mg (range 1.95 to 11.91 mU/mg). Residual VLCAD activity of 4 patients with confirmed VLCAD-deficiency was between 0.3 and 1.1 %. Heterozygous *ACADVL* mutation carriers showed residual VLCAD activities of 23.7 to 54.2 %.

MCAD activity in controls was 2.38 ± 0.18 mU/mg. In total, 28 patients with suspected MCAD-deficiency were assayed. Nearly all patients with residual MCAD activities below 2.5% were homozygous 985A>G carriers. MCAD-deficient patients with one other than the 985A>G mutation had higher MCAD residual activities, ranging from 5.7 to 13.9%. All patients with the 199T>C mutation had residual activities above 10%.

Conclusions: Our newly developed LC-MS/MS method is able to provide ample sensitivity to correctly and rapidly determine MCAD and VLCAD residual activity in human lymphocytes. Importantly, based on measured MCAD residual activities in correlation with genotype, new insights were obtained on the expected clinical phenotype.

Introduction

If undiagnosed and untreated, fatty acid oxidation defects (FAOD), such as medium- and very long-chain acyl-CoA dehydrogenase (MCAD and VLCAD, respectively) deficiency, are associated with high morbidity and mortality (Wanders et al. 1999). Clinical presentation of VLCAD deficiency may include cardiomyopathy, metabolic encephalopathy, hypoglycaemia and rhabdomyolysis. MCAD deficiency is associated with hepatic symptoms that are predominantly related to intercurrent illnesses or prolonged fasting. Thus, early detection by newborn screening (NBS) is absolutely essential for achieving a more favourable outcome and indeed, disease mortality and morbidity is significantly reduced (Arnold et al. 2009a; Spiekerkoetter et al. 2009).

However, NBS for MCAD and VLCAD deficiency using tandem mass spectrometry screening identifies false-positive cases and the outcome of the expanded NBS program in Germany, including MCAD and VLCAD deficiency, was recently assessed by Schulze *et al.* demonstrating that the positive predictive values for MCAD and VLCAD deficiency were 25.8 and 3.1 %, respectively (Schulze et al. 2003). In contrast to medium-chain acylcarnitines, mild accumulation of long-chain acylcarnitines is observed in healthy children due to activated mitochondrial fatty acid oxidation during catabolism (Schymik et al. 2006). Furthermore, VLCAD deficient patients may well present with normal confirmatory secondary screening after day 3 of life during anabolism (Browning et al. 2005). Taken together, each newborn with an acylcarnitine profile suggestive of MCAD or VLCAD deficiency needs further confirmatory diagnosis. Genotyping for prevalent mutations, i.e. the 985A>G mutation in case of MCAD deficiency, may represent rapid and cost-effective confirmatory techniques. However, the use of extended, and thus both time-consuming and expensive, mutation screening was reported to be necessary, particularly for suspected MCAD deficient patients who are not of European descent, as recently reviewed by Leonard *et al.* (Leonard and Dezateux 2009). Likewise, great molecular heterogeneity in VLCAD deficiency (Arnold et al. 2009b) makes extended mutation screening obligatory in most cases. Genotyping does, therefore, not always constitute a swift and cost-effective confirmatory diagnostic technique for a suspected FAOD in all cases.

In this paper we present a novel, cost-effective and rapid method for the measurement of MCAD and VLCAD activity in human lymphocytes to correctly identify patients and to confirm disease. In addition, we correlate residual MCAD activities with genotype in MCAD deficiency.

Methods

Participants

Whole blood specimens were obtained from subjects with biochemical indication of MCAD or VLCAD deficiency, as identified in NBS, their parents and siblings. As the determination of MCAD and/or VLCAD residual activity constitutes a routine procedure performed by our laboratory, not being part of a scientific study in any way or form, informed consent from subjects, or their parents, was obtained by the referring physicians in oral fashion. The study was performed according to the rules of the Declaration of Helsinki and approved under study number 3230 by the ethical review board of Heinrich-Heine-University Düsseldorf.

Reagents

Palmitoleoyl-CoA (C16:1-CoA), palmitoyl-CoA (C16:0-CoA) and octanoyl-CoA (C8:0-CoA) were purchased from Sigma (Deisenhofen, Germany) as Li^+ salts and stored at -20 °C. Ferrocenium hexafluorophosphate was obtained from Sigma-Aldrich (Deisenhofen, Germany).

Procedures

Isolation of lymphocytes from blood samples

Lymphocytes were isolated from 2 mL of whole blood using Ficoll-Paque™ Plus (Amersham) and Leucosep® (Greiner Bio One) tubes as described previously (Liebig et al. 2006).

VLCAD and MCAD enzyme assay conditions

Lymphocytes were resuspended in medium (200 µM ferrocenium hexafluorophosphate in 100 mM Tris-HCl or 10 mM NH_4Ac for HPLC and LC-MS/MS analysis, respectively) to a final protein concentration of 30 $\mu g \cdot mL^{-1}$. Duplicate enzyme reactions for 5 min. at 37°C and pH=8.0 were started by adding 4 µL of 5 mM C16:0-CoA as substrate to a total reaction volume of 100 µL. For HPLC analysis, quenching was achieved by adding 10 µL of 2 M HCl, followed by 5 min. of incubation on ice and neutralization with 2 M KOH. For LC-MS/MS analysis, the reaction was quenched by adding 100 µL of –20°C acetonitrile. Subsequently, proteins were removed by precipitation at 13000 rpm for 5 min. and by filtering the supernatant through 96-well 1.2 µm filter plates (Millipore, Schwalbach, Germany). C16:0-CoA was replaced by C8:0-CoA in the MCAD assay, at a concentration of 5 mM.

High-pressure-liquid-chromatography analysis

VLCAD assay products, C16:1-CoA and C16:OH-CoA, and the C16:0-CoA substrate were measured based on a method published by Woldegiorgis *et al.* (Woldegiorgis et al. 1985). Briefly, a Phenomenex C18(2) Luna column (250 mm x 4.6 mm x 5 µm) in combination with a C18 Luna guard column were used. The two mobile-phase solvents were acetonitrile and 25 mM KH_2PO_4, pH=6.9. Following 2 min. of 70 % KH_2PO_4 and 30 5 ACN, a three-step linear gradient was used; 1) 70 % KH_2PO_4 reduced to 60 % and 30 % acetonitrile increased to 40 % over 5 min. 2) 60 % KH_2PO_4 reduced to 54 % and 40 % acetonitrile increased to 46 % over 9 min. 3) 54 % KH_2PO_4 reduced to 38 % and 46 % acetonitrile increased to 62 % over 5 min. Next, the 38 % KH_2PO_4/62 % acetronitrile mixture was run for 5 min. Finally, the system was equilibrated for 5 min. The injection volume was 10 µL at a flow rate of 1.5 mL/min. The acyl-CoA esters were detected at 254 nm and 16:1-CoA, C16:OH-CoA and C16:0-CoA eluted at 11,6; 16,6 and 17,5 min, respectively. Quantitation was based on peak areas, with the initial substrate C16:0-CoA amount set to 20 nmol.

Tandem mass spectrometry analysis

In quenched VLCAD and MCAD assay samples, C16:1-CoA; C16:OH-CoA; C16:0-CoA and C8:1-CoA; C8:OH-CoA; C8:0-CoA, respectively, were quantified by LC-MS/MS. A Waters 2795 Alliance HPLC system (Waters, Milford, UK), equipped with a thermostated autosampler, was used for solvent delivery and sample introduction. Assay samples were placed in a cooled sample tray (15 °C) and 5 µL was injected onto a C18(2) Phenomenex Luna column (100 x 2.0 mm x 3µm). C16:OH-CoA, C16:1-CoA and C16:0-CoA were eluted isocratically with 55 % (v/v) acetonitrile in 10 mM NH_4Ac (pH was not adjusted and was approx. 6.7) at a flow rate of 200 µL/min. Similarly, C8:OH-CoA, C8:1-CoA and C8:0-CoA were eluted isocratically with 30 % (v/v) acetonitrile in 10 mM NH_4Ac. The eluate was delivered into a Quattro Micro MS/MS (Micromass, Cambridge, UK) with an ESI probe in positive ion mode. Injection interval was 5 min. Nitrogen was used as drying gas at 650 L/h. Collision energy was 35 eV and argon was used as collision gas. Declustering potential was 45 V and ion source temperature was 100°C. Compounds were detected in the multiple reaction monitoring (MRM) mode with the following mass transitions for the VLCAD assay: C16:OH-CoA, m/z 1022.4 → 515.2, C16:1-CoA, m/z 1004.4 → 497.2 and C16:0-CoA, m/z 1006.4 → 499.2, respectively, and C8:OH-CoA, m/z 910.4 → 403.2, C8:1-CoA, m/z 892.4 → 385.3 and C8:0-CoA, m/z 894.4 → 387.2, respectively, for the MCAD assay.

Quantitation was based on peak area ratios, with the initial substrate C16:0-CoA or C8:0-CoA amount set to 20 nmol.

Identification of mutations in the ACADM and ACADVL gene

Genomic DNA from whole blood was isolated using DNA Mini kit (Qiagen, Hilden, Germany). Polymerase chain reaction (PCR) was used to amplify all twelve *ACADM* exons, including part of the flanking intron sequences as previously described (Andresen et al. 1997). *ACADVL*, containing 20 exons, was analyzed according to Spiekerkoetter *et al*. (Liebig et al. 2006). The amplified DNA fragments were separated by electrophoresis and extracted (QIAquick Gel Extraction kit, Quiagen). PCR products were subjected to DNA sequence analysis using the Cycle sequencing kit, (Applied Biosystems, Weiterstadt, Germany). Analysis was performed using an Applied Biosystems Prism 310 Genetic Analyser.

Statistical methods

Data were acquired and analysed using MassLynx NT v4.0 (Micromass, UK). Reported data are presented as means ± standard error of the mean (SEM). Statistical analyses were performed using Student's *t* test. Differences between means were considered significant if $p < 0.05$.

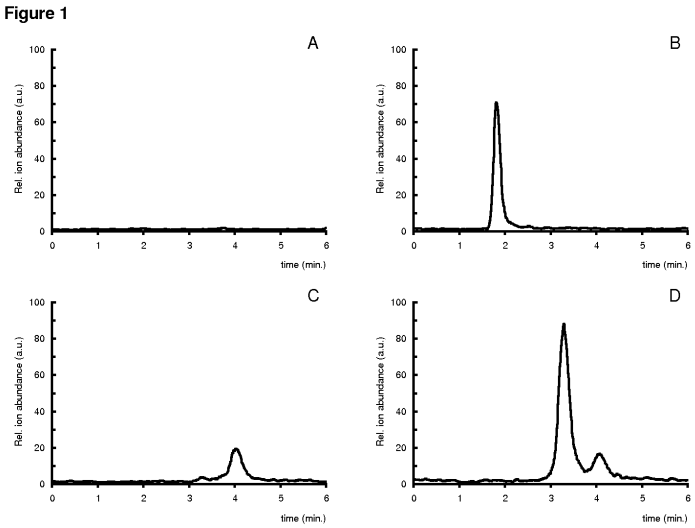


Figure 1: Multiple Reactant Monitoring (MRM) chromatograms of quenched VLCAD assay samples. C16:OH-CoA, m/z 1022.4 → 515.2 (A and B) and C16:1-CoA, m/z 1004.4 → 497.2 (C and D) after 0 (A and C) and 5 (B and D) min. of incubation.

Results

Characterization and validation of the acyl-CoA dehydrogenase assays

Representative VLCAD assay multiple reactant monitoring chromatograms are shown in Figure 1. The sample was either quenched instantly by adding –20°C acetonitrile, subsequent to substrate (C16:0-CoA) addition (Figure 1, A and B), or was incubated for 5 minutes at 37°C (Figure 1, C and D). The hydroxyl-CoA product eluted first (C16:OH-CoA fragment ion, m/z 1022.4 → 515.2) at 1.9 min. and the enoyl-CoA peak (C16:1-CoA fragment ion, m/z

1004.4 → 497.2) eluted at 3.2 min. The observed C16:1-CoA peak eluting at 4.0 min. was independent of assay incubation time or sample amount and was solely dependent on palmitoyl-CoA substrate concentration, most likely identifying it as C16:1-*cis*-9-CoA impurity in the commercial palmitoyl-CoA preparation. As enoyl-CoA products are rapidly converted into hydroxyacyl-CoA by matrix 2-enoyl-hydratases, in order to avoid product inhibition *in-vivo* (Powell et al. 1987; Davidson and Schulz 1982), it was critical to include hydroxyacyl-CoA as acyl-CoA dehydrogenase product to avoid underestimation of catalytic turnover.

Figure 2

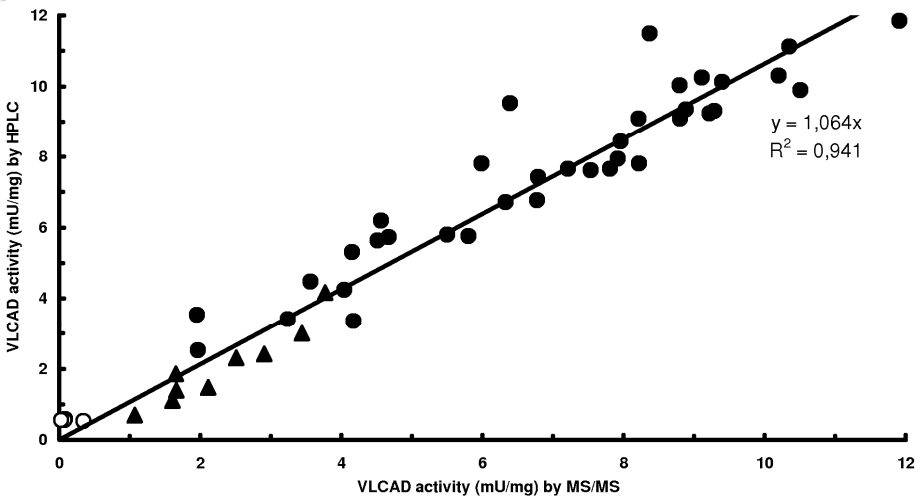


Figure 2: Comparison of results of the standard HPLC assay and the LC-MS/MS assay from the same lymphocyte sample. Total number of subjects tested was 52. (●) Normal controls. (▲) Confirmed heterozygous *ACADVL* mutation carriers. (○) Confirmed homozygous *ACADVL* mutation carriers.

The oxidation products in the MCAD assay, C8:OH-CoA and C8:1-CoA, and the added substrate C8:0-CoA had fragment masses of m/z 910.4 → 403.2, 892.4 → 385.3 and 894.4 → 387.2, respectively. MCAD and VLCAD activities in lymphocytes were linear up to 20 min, proportional to the amount of protein added in the range of 10 to 50 µg and were independent of ferrocenium hexafluorophosphate electron acceptor concentrations above 50 µM.

The inter-assay variation (CV) for the HPLC and LC-MS/MS method were determined by measuring the VLCAD activity in the same control lymphocytes on 8 different days and were

9 % and 13 %, respectively. To determine the intra-assay variation (CV), VLCAD activity was measured in one control lymphocyte sample in triplicate on two consecutive days. Intra-assay variations were 3 % and 4 % for the HPLC and LC-MS/MS method, respectively.

Table 1: Octanoyl-CoA oxidation, plasma medium-chain acylcarnitine levels and gene analysis of both *ACADM* alleles in subjects with suspected MCAD-deficiency.

Case	MCAD (%)	Allel 1	Allel 2	C8:0 I (µM)	C8:0 II (µM)
1	0.4%	985A > G	985A > G	21.00	6.30
2	0.5%	985A > G	985A > G	12.50	3.13
3	0.5%	985A > G	985A > G	19.70	2.64
4	0.6%	245ins > T	IVS9+2T>C[5]	6.00	
5	1.0%	985A > G	985A > G	0.97	0.85
6	1.1%	985A > G	347G > A	3.75	4.76
7	1.1%	985A > G	985A > G		
8	1.3%	245ins > T	IVS9+2T>C[5]	7.73	
9	1.7%	985A > G	985A > G	8.70	2.80
10	1.7%	985A > G	985A > G	12.00	1.42
11	2.3%	985A > G	985A > G	3.40	2.30
12	5.7%	985A > G	IVS[6]	2.80	1.48
13	6.3%	985A > G	IVS[6]	7.23	2.51
14	7.5%	823A > G	823A > G	1.90	2.57
15	9.3%	157C > T	157C > T		1.65
16	10.2%	985A > G	199T > C	4.27	
17	11.8%	985A > G	199T > C	1.82	
18	13.9%	1140ins > G	199T > C		0.48
19[7]	24.5%	wild-type	wild-type		
20[3]	27.2%	985A > G	wild-type		
21[3]	30.8%	wild-type	IVS[6]		
22[7]	33.5%	985A > G	wild-type		
23[4]	33.6%	985A > G	wild-type		
24[7]	47.5%	wild-type	wild-type		
25[4]	53.4%	199T > C	wild-type		
26[7]	69.9%	wild-type	wild-type		
27[7]	76.1%	wild-type	wild-type		
28[7]	76.5%	wild-type	wild-type		

Case 1 to 18 were identified in NBS, with the exception of 4 and 8.

[1]Relative residual MCAD enzyme activities are presented as a percentage of the mean of lymphocytes from healthy control (2.38 nmol · min^{-1} · mg^{-1}, n = 6); [2]First octanoylcarnitine specimen (C8:0 I) obtained on day 2 – 5 of life and subsequent repeat specimen (C8:0 II) are shown in µmol · L^{-1}, cut-off was set at 0.30; [3]Parents of 12; [4]Parents of 16; [5]Intervening sequences: IVS2-32C>G, IVS3+10T>C, IVS5+32C>G, IVS7-22C>A; [6]Intervening sequences: IVS2-32C>G, IVS3+10T>C, IVS5+32C>G, IVS6-14A>G and IVS7-22C>A; [7]subjects with clinical suspicion of MCAD deficiency

We compared results obtained with our newly developed LC-MS/MS VLCAD assay with VLCAD activities determined by HPLC. In a direct comparison of VLCAD activities in human lymphocytes, the LC-MS/MS method correlates well with the HPLC assay (Figure 2). We observed that correlation between the established HPLC and newly developed LC-MS/MS method was excellent ($r = 0.941$) and S_{yx} was ± 0.804 (Figure 2).

Determination of residual VLCAD activities in patients

In total, 52 subjects were measured with our LC-MS/MS VLCAD assay. Four were identified as VLCAD deficient patients and 9 were heterozygous carriers of an *ACADVL* mutation (Figure 2). VLCAD activities measured by LC-MS/MS in the samples collected from normal subjects (n = 36) ranged from 1.95 to 11.91 nmol · min^{-1} · mg $protein^{-1}$, with a mean ± SEM value of 6.95 ± 0.42 nmol · min^{-1} · mg $protein^{-1}$ (Figure 2). Average residual VLCAD activities of four confirmed patients, being homozygous carriers of an *ACADVL* mutation, was 0.13 ± 0.07 nmol · min^{-1} · mg $protein^{-1}$, representing residual activities between 0.02 and 0.34 nmol · min^{-1} · mg $protein^{-1}$ (Figure 2). In confirmed heterozygous carriers of an *ACADVL* mutation, residual VLCAD activity ranged from 1.07 to 3.77 nmol · min^{-1} · mg $protein^{-1}$. VLCAD activity measurements with HPLC revealed comparable results, but homozygous *ACADVL* mutation carriers had a higher average residual VLCAD activity of 0.55 ± 0.01 nmol · min^{-1} · mg $protein^{-1}$ (Figure 2).

Determination of residual MCAD activities in patients

MCAD activities measured by LC-MS/MS in the samples collected from normal subjects (n = 6) ranged from 1.82 to 2.78 nmol · min^{-1} · mg $protein^{-1}$, with a mean ± SE value of 2.38 ± 0.18 nmol · min^{-1} · mg $protein^{-1}$. Table 1 lists 28 examined cases that were assayed for MCAD activity, parents of newborns are highlighted. With the exception of cases 4, 6 and 8 all patients with residual MCAD activities below 2.5 % were homozygous carriers of the 985A>G mutation. MCAD deficient patients with other mutations had higher MCAD residual activities, ranging from 5.7 to 13.9 %. Patients carrying one 199T>C mutation presented with residual activities above 10 %. Confirmed 985A>G heterozygotes (cases 20, 22 and 23) had residual MCAD activities above 24 % of controls (Table 1).

Genotypes of MCAD-deficient patients

Mutation analysis revealed that 8 out of 18 patients were 985A>G homozygotes (Table 1). Cases 4 and 8 presented with an insertion at position 245, as reported previously by Gregersen *et al.* and Maier *et al.* (Gregersen et al. 2008; Maier et al. 2005), and were heterozygous for a splicing mutation (IVS9+2T>C). In addition, multiple other intronic sequence variants (IVS)

were observed (IVS2-32C>G, IVS3+10T>C, IVS5+32C>G, IVS7-22C>A). Case 6, in addition to mutation 985A>G, carried the 347G>A mutation, a mutation previously described as severe (Andresen et al. 2001; Gregersen et al. 2008). To the best of our knowledge, case 14 carried a novel mutation and was homozygous for 823A>G. Case 15 was a homozygous carrier of the 157C>T mutation, reported earlier (Andresen et al. 2001; Gregersen et al. 2008; Maier et al. 2005). Cases 16 and 17 were compound heterozygous for mutations 985A>G and 199T>C, as reported earlier (Andresen et al. 1997; Gregersen et al. 2008). An insertion at position 1140 was identified in case 18 and was also previously not reported. In cases 18, 24, 26-28 a FAOD was clinically suspected but the subjects turned out not to carry a mutation on both *ACADM* alleles. Case 21, parent of case 12, had multiple heterozygous intervening sequences (IVS2-32C>G, IVS3+10T>C, IVS5+32C>G, IVS6-14A>G and IVS7-22C>A). Cases 12 and 13 originated from different families and were both heterozygous carriers of the same above described intervening sequences, in addition to their classical 985A>G mutation (Table 1).

Correlation of genotype with MCAD residual activity and C8:0-carnitine concentration

As indicated in Table 1, initial C8:0-carnitine does not always correlate with genotype and residual enzyme activity. However, a high C8:0-carnitine above >10 µM was only observed in MCAD deficiency patients with residual MCAD activities below 2 % of controls and the homozygous 985A>G mutation (Figure 3).

Figure 3

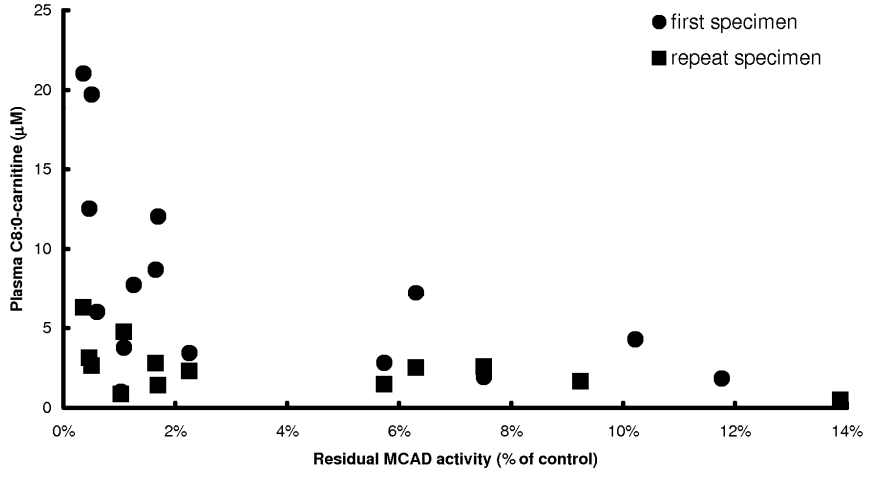


Figure 3: Plasma octanoyl-carnitine (C8:0) concentrations determined in dried blood spots during initial newborn screening and subsequent follow-up from patients carrying two confirmed *ACADM* mutations.

Discussion

Our newly developed LC-MS/MS method for the determination of MCAD- and VLCAD-enzyme activities in human lymphocytes is able to provide ample sensitivity to correctly and rapidly confirm diagnosis of the respective disorder in individuals identified by newborn screening. This technique is of great importance because long-chain acylcarnitines are elevated in healthy children or VLCAD heterozygotes, due to activated fatty acid oxidation in the first 2-3 days of life, and are thus interfering with NBS results. In case of MCAD deficiency, this assay also offers the possibility to predict severity of expected clinical phenotype based on residual MCAD activities and correlating genotype.

As the LC-MS/MS analytical apparatus is available in all NBS laboratories worldwide, it was important to develop an analysis method that can be used with available NBS instruments. This newly developed method thus offers the possibility to determine MCAD or VLCAD activity in one single setup, without exchanging columns, and lacking complex gradients.

Residual VLCAD activity in VLCAD deficient patient lymphocytes

Enzyme activities of VLCAD in human lymphocytes from healthy controls were 6.95 ± 0.42 nmol · min^{-1} · mg $protein^{-1}$ (n = 36) and are similar to control values reported previously by other laboratories (Wanders and Ijlst 1992; Aliefendioglu et al. 2007; Voermans et al. 2006). Figure 2 clearly shows that heterozygous carriers can not be discriminated from healthy controls in all cases. However, most importantly, VLCAD deficiency patients confirmed by molecular analysis of the *ACADVL* gene can unequivocally be identified based on the determination of residual VLCAD activity in isolated lymphocytes.

Correlation of residual MCAD activity with ACADM genotype

Unlike VLCAD deficiency with molecular heterogeneity, MCAD deficiency possesses the prevalent 985A>G mutation and, since NBS, the less common 199T>C mutation. The total number of individual *ACADM* mutations reported so far is approx. 25, as reviewed in (Gregersen et al. 2008). We therefore, attempted to correlate residual MCAD-activities with our *ACADM* genotyping results. This was not pursued for VLCAD activities and corresponding mutations in the *ACADVL* gene, also because of much greater molecular heterogeneity and due to the limited number of observed homozygous *ACADVL* mutation carriers.

MCAD activities in healthy controls were 2.38 ± 0.18 nmol · min^{-1} · mg $protein^{-1}$, (n = 6) being in agreement with previous reports (Wanders and Ijlst 1992; Niezen-Koning et al. 1991; Duran et al. 1992). The MCAD assay was applied in lymphocyte samples from 28 subjects with suspected MCAD deficiency. In 18 individuals, diagnosis was confirmed by mutational analysis (Table 1). All patients with the homozygous, classical 985A>G mutation had residual MCAD activities below 2.5 %, therefore well in line with previous reports that identify the homozygous status of the 985A>G mutation as severe clinical phenotype (Andresen et al. 1997; Andresen et al. 2001). The 985A>G mutation was the prevalent MCAD mutation before the NBS era and patients were identified because of hypoglycaemia or hepatopathy (Andresen et al. 2001). In addition to mutation 985A>G, one MCAD deficient patient was carrier of the 347G>A mutation, a mutation previously described as severe (Andresen et al. 2001; Gregersen et al. 2008), this again is in excellent agreement with a low residual MCAD activity of 1.1 %. The more recent 199T>C mutation (Gregersen et al. 2008) was identified in a significant number of children since implementation of NBS and is considered to induce a much milder MCAD deficiency variant (Gregersen et al. 2008). In our cohort, this mutation was identified three times, two in combination with the 985A>G mutation, and all three resulted in significantly higher residual MCAD activities of approx. 11 %, as compared to patients being homozygous for the 985A>G mutation. Also demonstrating that MCAD deficiency severity, in case of compound heterozygosity, is determined by the mutation that is known to be the mildest of the two, in this case the 199T>C mutation (Gregersen et al. 2008). In addition, patients with a heterozygous 245insT mutation and a second splicing IVS9+2T>C mutation had comparably lower residual activities of < 2.5 %. For the homozygous 157C>T mutation, reported earlier by (Andresen et al. 2001; Gregersen et al. 2008; Maier et al. 2005) to result in a mild clinical phenotype, enzymatic analysis now revealed that this mutation leads to 9.3 % residual MCAD activity. Interestingly, two unrelated cases presented with the 985A>G mutation plus identical mutations in intervening *ACADM* sequences (IVS2-32C>G, IVS3+10T>C, IVS5+32C>G, IVS6-14A>G and IVS7-22C>A) and both had residual MCAD activities of 6 %. Heterozygous carriers of the 985A>G mutation without a second mutation (cases 20, 22 and 23) had residual MCAD activities of 24 % or higher (Table 1).

The use of plasma octanoyl-carnitine levels in newborn screening for MCAD deficiency

In our hands, measured plasma octanoyl-carnitine (C8:0) levels, based on the initial NBS DBS, indeed functions as sensitive and specific marker for MCAD deficiency (Fig. 3). However, the C8:0-carnitine values measured are unable to discriminate between MCAD deficiency of different severity. This also applies to the repeat specimen upon confirmatory

second screening, being well in line with a previous report from Chace and co-workers (Chace et al. 1997).

Possible limitations of this study

The use of isolated peripheral blood lymphocytes may well not be fully representative for all individual organs that are differentially afflicted by MCAD or VLCAD deficiency. Of note, studies in fibroblasts would obviously present similar limitations. Furthermore, the use of ferrocenium as artificial oxidizing agent in our *in-vitro* assay, instead of electron transfer flavoprotein *in-vivo*, may possibly obscure alterations in MCAD and VLCAD activity induced by mutations that impair flavin adenine dinucleotide binding and/or electron transfer flavoprotein interaction.

Conclusions

Taken together, we have developed a new assay that allows fast and reliable determination of MCAD and VLCAD residual activity in human lymphocytes. This method takes advantage of LC-MS/MS detection and can be implemented in any NBS laboratory using LC-MS/MS technique. Both methods provide reference values for MCAD and VLCAD enzyme activity in lymphocytes from healthy controls that correspond well with published rates in literature. Importantly, both methods provide ample sensitivity to discriminate not only patients from healthy individuals but, in case of MCAD deficiency, this method also offers the possibility to differentiate between clinical phenotypes of different severity based on residual MCAD activities, in line with their genotype.

References

1. Aliefendioglu D, Dursun A, Coskun T, Akcoren Z, Wanders RJ, Waterham HR (2007) A newborn with VLCAD deficiency Clinical, biochemical, and histopathological findings. Eur J Pediatr

2. Andresen BS, Bross P, Udvari S, Kirk J, Gray G, Kmoch S, Chamoles N, Knudsen I, Winter V, Wilcken B, Yokota I, Hart K, Packman S, Harpey JP, Saudubray JM, Hale DE, Bolund L, Kolvraa S, Gregersen N (1997) The molecular basis of medium-chain acyl-CoA dehydrogenase (MCAD) deficiency in compound heterozygous patients: is there correlation between genotype and phenotype? Hum Mol Genet 6:695-707

3. Andresen BS, Dobrowolski SF, O'Reilly L, Muenzer J, McCandless SE, Frazier DM, Udvari S, Bross P, Knudsen I, Banas R, Chace DH, Engel P, Naylor EW, Gregersen N (2001) Medium-chain acyl-CoA dehydrogenase (MCAD) mutations identified by MS/MS-based prospective screening of newborns differ from those observed in patients with clinical symptoms: identification and characterization of a new, prevalent mutation that results in mild MCAD deficiency. Am J Hum Genet 68:1408-1418

4. Arnold GL, Van HJ, Freedenberg D, Strauss A, Longo N, Burton B, Garganta C, Ficicioglu C, Cederbaum S, Harding C, Boles RG, Matern D, Chakraborty P, Feigenbaum A (2009a) A Delphi clinical practice protocol for the management of very long chain acyl-CoA dehydrogenase deficiency. Mol Genet Metab 96:85-90

5. Arnold GL, Van HJ, Freedenberg D, Strauss A, Longo N, Burton B, Garganta C, Ficicioglu C, Cederbaum S, Harding C, Boles RG, Matern D, Chakraborty P, Feigenbaum A (2009b) A Delphi clinical practice protocol for the management of very long chain acyl-CoA dehydrogenase deficiency. Mol Genet Metab 96:85-90

6. Browning MF, Larson C, Strauss A, Marsden DL (2005) Normal acylcarnitine levels during confirmation of abnormal newborn screening in long-chain fatty acid oxidation defects. J Inherit Metab Dis 28:545-550

7. Chace DH, Hillman SL, Van Hove JL, Naylor EW (1997) Rapid diagnosis of MCAD deficiency: quantitative analysis of octanoylcarnitine and other acylcarnitines in newborn blood spots by tandem mass spectrometry. Clin Chem 43:2106-2113

8. Davidson B, Schulz H (1982) Separation, properties, and regulation of acyl coenzyme A dehydrogenases from bovine heat and liver. Arch Biochem Biophys 213:155-162

9. Duran M, Cleutjens CB, Ketting D, Dorland L, de Klerk JB, van Sprang FJ, Berger R (1992) Diagnosis of medium-chain acyl-CoA dehydrogenase deficiency in lymphocytes and liver by a gas chromatographic method: the effect of oral riboflavin supplementation. Pediatr Res 31:39-42

10. Gregersen N, Andresen BS, Pedersen CB, Olsen RK, Corydon TJ, Bross P (2008) Mitochondrial fatty acid oxidation defects-remaining challenges. J Inherit Metab Dis

11. Leonard JV, Dezateux C (2009) Newborn screening for medium chain acyl CoA dehydrogenase deficiency. Arch Dis Child 94:235-238

12. Liebig M, Schymik I, Mueller M, Wendel U, Mayatepek E, Ruiter J, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Neonatal screening for very long-chain acyl-coA dehydrogenase deficiency: enzymatic and molecular evaluation of neonates with elevated C14:1-carnitine levels. Pediatrics 118:1065-1069

13. Maier EM, Liebl B, Roschinger W, Nennstiel-Ratzel U, Fingerhut R, Olgemoller B, Busch U, Krone N, Kries R, Roscher AA (2005) Population spectrum of ACADM genotypes correlated to biochemical phenotypes in newborn screening for medium-chain acyl-CoA dehydrogenase deficiency. Hum Mutat 25:443-452

14. Niezen-Koning KE, Chapman TE, Mulder IE, Smit GP, Reijngoud DJ, Berger R (1991) Determination of medium chain acyl-CoA dehydrogenase activity in cultured skin fibroblasts using mass spectrometry. Clin Chim Acta 199:173-184

15. Powell PJ, Lau SM, Killian D, Thorpe C (1987) Interaction of acyl coenzyme A substrates and analogues with pig kidney medium-chain acyl-coA dehydrogenase. Biochemistry 26:3704-3710

16. Schulze A, Lindner M, Kohlmuller D, Olgemoller K, Mayatepek E, Hoffmann GF (2003) Expanded newborn screening for inborn errors of metabolism by electrospray

ionization-tandem mass spectrometry: results, outcome, and implications. Pediatrics 111:1399-1406

17. Schymik I, Liebig M, Mueller M, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Pitfalls of neonatal screening for very-long-chain acyl-CoA dehydrogenase deficiency using tandem mass spectrometry. J Pediatr 149:128-130

18. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de KH, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis

19. Voermans NC, van Engelen BG, Kluijtmans LA, Stikkelbroeck NM, Hermus AR (2006) Rhabdomyolysis caused by an inherited metabolic disease: very long-chain acyl-CoA dehydrogenase deficiency. Am J Med 119:176-179

20. Wanders RJ, Ijlst L (1992) Fatty acid beta-oxidation in leukocytes from control subjects and medium-chain acyl-CoA dehydrogenase deficient patients. Biochim Biophys Acta 1138:80-84

21. Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, Ijlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. J Inherit Metab Dis 22:442-487

22. Woldegiorgis G, Spennetta T, Corkey BE, Williamson JR, Shrago E (1985) Extraction of tissue long-chain acyl-CoA esters and measurement by reverse-phase high-performance liquid chromatography. Anal Biochem 150:8-12

Chapter 5

Pre-exercise medium-chain triglyceride application prevents acylcarnitine accumulation in skeletal muscle from very-long-chain acyl-CoA-dehydrogenase-deficient mice

S. Primassin, S. Tucci, D. Herebian, A. Seibt, L. Hoffmann, F. ter Veld and U. Spiekerkoetter

J Inherit Metab Dis. 2010 Jun;33(3):237-46

Abstract

Dietary modification with medium-chain triglyceride (MCT) supplementation is one crucial way of treating children with long-chain fatty acid oxidation disorders. Recently, supplementation prior to exercise has been reported to prevent muscular pain and rhabdomyolysis. Systematic studies to determine when MCT supplementation is most beneficial have not yet been undertaken. We studied the effects of an MCT-based diet compared with MCT administration only prior to exercise in very-long-chain acyl-CoA dehydrogenase (VLCAD) knockout (KO) mice. VLCAD KO mice were fed an MCT-based diet in same amounts as normal mouse diet containing long-chain triglycerides (LCT) and were exercised on a treadmill. Mice fed a normal LCT diet received MCT only prior to exercise. Acylcarnitine concentration, free carnitine concentration, and acyl-coenzyme A (CoA) oxidation capacity in skeletal muscle as well as hepatic lipid accumulation were determined. Long-chain acylcarnitines significantly increased in VLCAD-deficient skeletal muscle with an MCT diet compared with an LCT diet with MCT bolus prior to exercise, whereas an MCT bolus treatment significantly decreased long-chain acylcarnitines after exercise compared with an LCT diet. C8-carnitine was significantly increased in skeletal muscle after MCT bolus treatment and exercise compared with LCT and long-term MCT treatment. Increased hepatic lipid accumulation was observed in long-term MCT-treated KO mice. MCT seems most beneficial when given in a single dose directly prior to exercise to prevent acylcarnitine accumulation. In contrast, continuous MCT treatment produces a higher skeletal muscle content of long-chain acylcarnitines after exercise and increases hepatic lipid storage in VLCAD KO mice.

Introduction

Very-long-chain acyl-coenzyme A (CoA) dehydrogenase (VLCAD, EC 1.3.99.13) deficiency (OMIM #201475) is an inherited but treatable disorder presenting with a severe early onset form of cardiomyopathy and Reye-like symptoms; a hepatic phenotype that usually expresses in infancy with recurrent hypoketotic hypoglycemia; and a milder, later-onset, myopathic form with episodic muscle weakness and rhabdomyolysis (Gregersen et al. 2004). It is also part of many newborn screening programs worldwide. VLCAD-deficient patients are usually asymptomatic at birth, but may become critically ill with prolonged fasting when lipolysis is induced, such as occurs during common childhood illnesses (Andresen et al. 1999). Patients with milder phenotypes may present during childhood or early adulthood with muscular pain, weakness, or rhabdomyolysis after increased energy demand, for example, with physical exercise (Gregersen et al. 2001). VLCAD is one of the enzymes catalyzing the first step of mitochondrial β-oxidation and is responsible for oxidation of long-chain (C14–C20) acyl-CoAs. Other enzymes, such as long-chain acyl-CoA dehydrogenase (LCAD), have partly overlapping substrate specificities (C10–C16; Wanders et al. 1999), but LCAD is not as highly expressed in most human tissues (Chegary et al. 2009). Medium-chain acyl-CoA dehydrogenase (MCAD) metabolizes medium-chain fatty acids (MCFA) (C6–C10), which are primarily the β-oxidation products brought about by long-chain fatty acid oxidation. Dietary fat mainly consists of long-chain fatty acids with C16–C20 chain lengths. VLCAD-deficient patients accumulate long-chain acylcarnitines due to insufficient oxidation of these long-chain acyl-CoAs. Consequently, medium-chain triglyceride (MCT) forms parts of dietary treatment in disorders of mitochondrial β-oxidation defects of long-chain fatty acids. In contrast to long-chain triglycerides (LCT), MCT can be metabolized in VLCAD-deficient patients because the specific enzymes for substrates of medium-chain lengths are completely functioning. In addition, absorption and transport of MCT into mitochondria is hypothesized to be less complicated compared with LCT, as MCT do not require esterification into acylcarnitines to cross the mitochondrial membranes and are more rapidly metabolized (Bach and Babayan 1982). Only recently it has been observed that patients with myopathic phenotypes profit especially from MCT supplementation directly prior to exercise to successfully prevent rhabdomyolysis and muscular pain due to increased energy demand. With this regimen, a biochemical study (Gillingham et al. 2006) demonstrated positive treatment effects in children with long-chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD) or mitochondrial trifunctional protein (TFP) deficiency. Another clinical study (Spiekerkoetter 2007) reported the positive

effect of MCT bolus treatment prior to exercise in a VLCAD-deficient patient, preventing myopathy and rhabdomyolysis. In humans, blood parameters serve for therapy monitoring, but previous studies have demonstrated that blood and tissue concentrations of various metabolites in fatty acid oxidation do not correlate (Primassin et al. 2008). Therefore, studies in an animal model are essential to analyze the metabolic changes in different tissues dependent on dietary modifications. As VLCAD knockout (KO) mice present a myopathic phenotype with exercise, similar to patients with impaired maximum running speed and reduced running distance (Cox et al. 2001; Exil et al. 2003), they are an appropriate model for the investigations outlined. In this study, we determined whether an LCT-based diet with MCT supplementation prior to exercise compared with an MCT-based diet is more beneficial in VLCAD KO mice. It has been previously shown that in the VLCAD KO mice, acylcarnitines accumulate in different tissues in situations of increased energy demand as a sign of activated fatty acid oxidation but impaired energy provision from fat (Spiekerkoetter et al. 2005). We measured acylcarnitine concentrations in skeletal muscle under the different dietary conditions as well as acyl-CoA oxidation capacity before and after exercise to determine β-oxidation efficiency after MCT supply. We also analyzed liver slices to determine how the supplied fat is further metabolized.

Materials and Methods

Reagents

Palmitoyl-CoA (C16-CoA) and octanoyl-CoA (C8-CoA) were purchased from Sigma-Alrich (Deisenhofen, Germany) as Li^+ salts and stored at -20 °C. Ferrocenium hexafluorophosphate was obtained from Sigma-Aldrich (Deisenhofen, Germany). The [2H3] carnitine (C0) and [2H3] C8- and C16-carnitine internal standards were obtained from Dr. Herman J. ten Brink (VU Medical Hospital, Amsterdam, Netherlands).

Animals

VLCAD KO mice were generated as described previously (Exil et al. 2003; Papaioannou VE and Johnson RS 1993; Spiekerkoetter et al. 2005) and were provided by Prof. Dr. Arnold Strauss, former Vanderbilt University Hospital, Nashville, TN, USA, now of the University of Cincinnati, College of Medicine, Cincinnati, OH, USA. Genotypes were determined by duplicate polymerase chain reaction (PCR) analysis.

Experiments were performed on fourth- to fifth-generation intercrosses of C57BL6+129sv VLCAD genotypes. Littermates served as controls and genotyping of mice was performed as described previously (Spiekerkoetter et al. 2005). At the age of 10-12 weeks mice of both genotypes were analyzed under well-fed, resting conditions and after short-term exercise. Exercised mice of the various treatment groups were last fed half an hour prior to exercise. Each group consisted of five mice (n = 5). All mice were weighed and sacrificed by cervical dislocation, the mice in the exercise group immediately after termination of exercise. Liver and skeletal muscle samples were rapidly removed, weighed and frozen immediately in liquid nitrogen. Blood samples were dried on a filter paper card. All animal studies were performed with the approval of the Heinrich-Heine-University Institutional Animal Care and Use Committee. Care of the animals was in accordance with the Heinrich-Heine-University Medical Centre and Institutional Animal Care and Use Committee guidelines.

Diet composition and supplementation

After being weaned, the first group of KO mice received a purified mouse diet containing LCT (subsequently referred to as LCT diet). The purified diet contained 5.1 % crude fat, corresponding to 13 % of metabolizable energy calculated with Atwater factors (ssniff® EF R/M Control, ssniff Spezialdiäten GmbH). With an approximate intake of 5 g food per day, the LCT intake for each mouse (body weight approximately 30 g) was estimated at 250 mg per day. The second group of KO mice received a similar diet (ssniff® EF R/M control, ssniff GmbH. Soest, Germany), but the diet contained 4.4 % crude fat as MCT (Ceres®MCT oil, basis GmbH, Oberpfaffenhofen, Germany) and 0.6 % crude fat as LCT, as essential soy bean oil, corresponding to approximately 13 % of total metabolizable energy calculated with Atwater factors. In these mice the amount of essential LCT accounted to approximately 30 mg per day and MCT to approximately 220 mg per day. This diet is subsequently referred to as the MCT diet. The third group of KO mice was fed on the LCT diet but additionally received an oral bolus of MCT half an hour prior to exercise. This diet is subsequently referred to as LCT diet with pre-exercise MCT bolus. The applied dose of MCT was calculated at 2 g/kg body weight of MCT oil, equalling approximately 60 mg. The recommended dosage for humans is 0.25-0.5 g/kg body weight (Spiekerkoetter et al. 2009) and was adjusted to a higher amount for mice due to handling of a small amount. This is in addition to an estimated daily intake of approximately 250 mg LCT. Carbohydrate and protein content were unmodified and corresponded to 65 % and 22 % of metabolizable energy, respectively.

Exercise protocol

As mice are nocturnal animals, treadmill running was performed during the dark cycle. Three-month-old wild-type (WT) and VLCAD KO animals were exercised 60 min on a Columbus Instruments Simplex II metabolic rodent treadmill consisting of four individual lanes without inclination and a shock plate incentive (3 Hz, 200 ms, 160 V, 1.5 mA). Mice were placed in an exercise chamber, and after an adaptation period of 15 min, initial belt speed was set to 4 m/min and increased every 5 min by 2 m/min to a maximum of 16 m/min. Mice were exercised until they displayed signs of exhaustion (> 2 s spent on the shocker plate without attempting to re-engage the treadmill) or the exercise was terminated after 60 min.

Analysis of carnitine and acylcarnitines

Analysis of carnitine and acylcarnitines was performed in blood and tissues as described previously (Primassin et al. 2008). Briefly, blood carnitine and acylcarnitines were extracted in methanol (100 %) from dried spots (equivalent to 25 µl of blood) and liver and skeletal muscle were lyophilized for 12 h in the presence of internal standards (16.25 nmol [$^2H_3$] carnitine, for carnitine and γ-butyrobetaine concentrations; 0.1 nmol [$^2H_3$]C8-carnitine, for C2-C12 carnitines; 0.05 nmol [$^2H_3$]C16-carnitine, for C14-C18 carnitines). The lyophilized tissues were pulverized and dissolved in 1 ml acetonitrile/water (ACN/H_2O) (80/20 % v/v). After homogenization and centrifugation the supernatant was dried. Finally, γ-butyrobetaine, carnitine, and acylcarnitines were analyzed by ESI-MS/MS as their butyl esters and resuspended in 100 µl ACN/H_2O (50/50 % v/v).

Tissue enzyme activity

Liver and skeletal muscle tissues were minced intensively in ice-cold 100 mmol/L ammonium acetate (NH_4Ac) (pH 8) using scissors and homogenized in an Eppendorf tube by five strokes (up and down) using a loosely fitting pestle rotating at 600 *rpm*. Large cell debris and nuclei were pelleted by centrifugation for 10 min at 2,500 *rpm* at 4 °C in an Eppendorf centrifuge and the supernatant was taken for further use in the assay. Next, tissue homogenates were taken up in assay medium [200 µmol/L ferrocenium hexafluorophosphate in 100 mmol/L NH_4Ac (pH 8)] at a final protein concentration of 20 µg/ml. Enzyme incubations were carried out for 10 min at 37 °C after addition of 20 nmol C8:0-CoA or C16:0-CoA as substrate to a total reaction volume of 100 µl to determine MCAD or VLCAD activity, respectively. The reaction was quenched by addition of 100 µl –20 °C ACN (100 %). Subsequently, proteins were removed by precipitation at 13.000 *rpm* in an Eppendorf centrifuge for 5 min and by filtering the supernatant through 96-well 1.2-µm filter plates (Millipore, Schwalbach, Germany). Finally, in quenched VLCAD and MCAD assay samples,

C16:1-CoA, C16:OH-CoA, C16:0-CoA and C8:1-CoA, C8:OH-CoA, C8:0-CoA, respectively, were quantified by liquid chromatography - tandem mass spectrometry (LC-MS/MS) according to a method described previously by ter Veld et al. (ter Veld et al. 2009). Quantitation was based on peak area ratios, with the initial substrate C16:0 (palmitoyl)-CoA or C8:0 (octanoyl)-CoA amount set to 20 nmol. All assays were carried out in duplicate.

Nile red images

Mouse liver was placed in Tissue-Tek O.T.C. (Sakura, Zoeterwoude, The Netherlands), rapidly frozen and stored at −70 °C. Prior to cutting, the frozen blocks were equilibrated to −20 °C, and then sectioned to a thickness of 10 μm in a cryostat. Liver slices were used to stain neutral lipids. The staining was performed with Nile red (0.2 mg/ml; Sigma-Aldrich, Schnelldorf, Germany) for 5 min at room temperature (Greenspan et al. 1985). Nile red fluorescence was measured using a Zeiss Apotome® and Axio vision software (Carl Zeiss, Jena, Germany) using a 63×/1.4 objective lens. This system provides an optical slice view reconstructed from fluorescent samples. Neutral lipid images were collected using filter set 10 (488010-9901-000). The excitation and emission wavelengths of 480 nm and 540 nm, respectively, were used to evidence the yellow-gold fluorescence emitted from neutral lipids (Fowler and Greenspan 1985).

Statistical analysis

Data were acquired and analyzed using MassLynx NT v4.0 (Micromass, UK). Data are expressed as the means ± standard error of the mean (SEM). Statistical analysis of differences between two means was assessed by Student's *t* test. Multiple means were compared by a two-way analysis of the variance (ANOVA) with a Bonferroni post hoc test (GraphPad Prism 4·0, San Diego, CA, USA). A probability level of $p<0{\cdot}05$ was regarded as significant.

Results

Body and tissue weights of the animals were similar within all groups. The average body weight of the mice was 28 g (± 1.73). With a set running speed of 16 m/min, VLCAD KO mice managed to run for 55 min (± 1.96) with the LCT diet and 50 min (± 1.74) with the MCT diet before exhaustion. VLCAD KO mice with the LCT diet with pre-exercise MCT bolus managed to run 60 min without exhaustion, the same as WT mice.

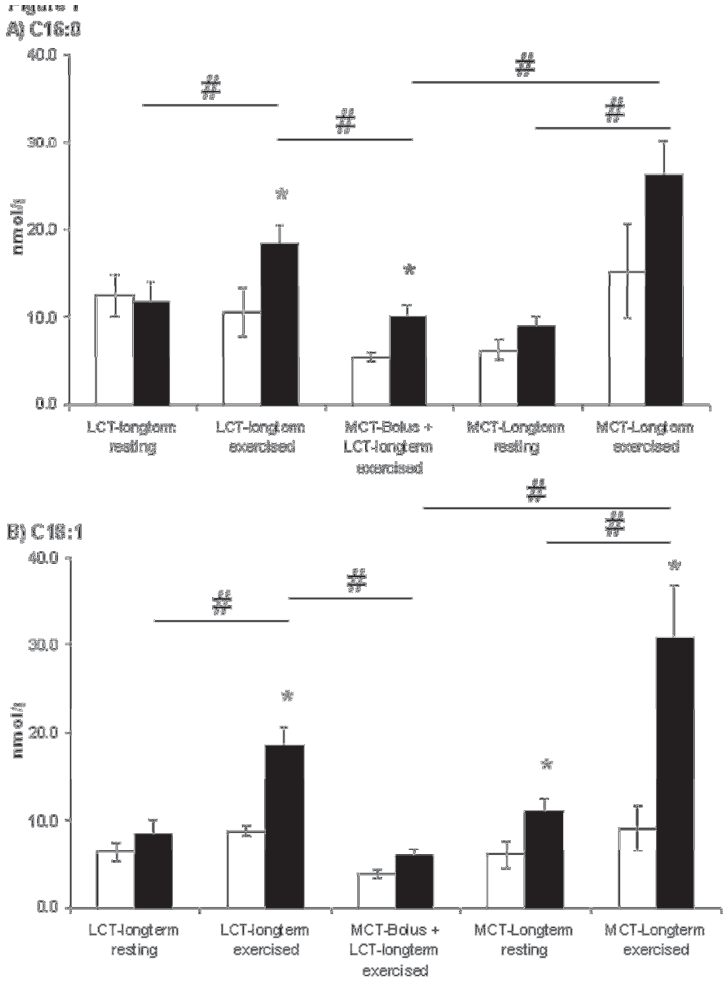
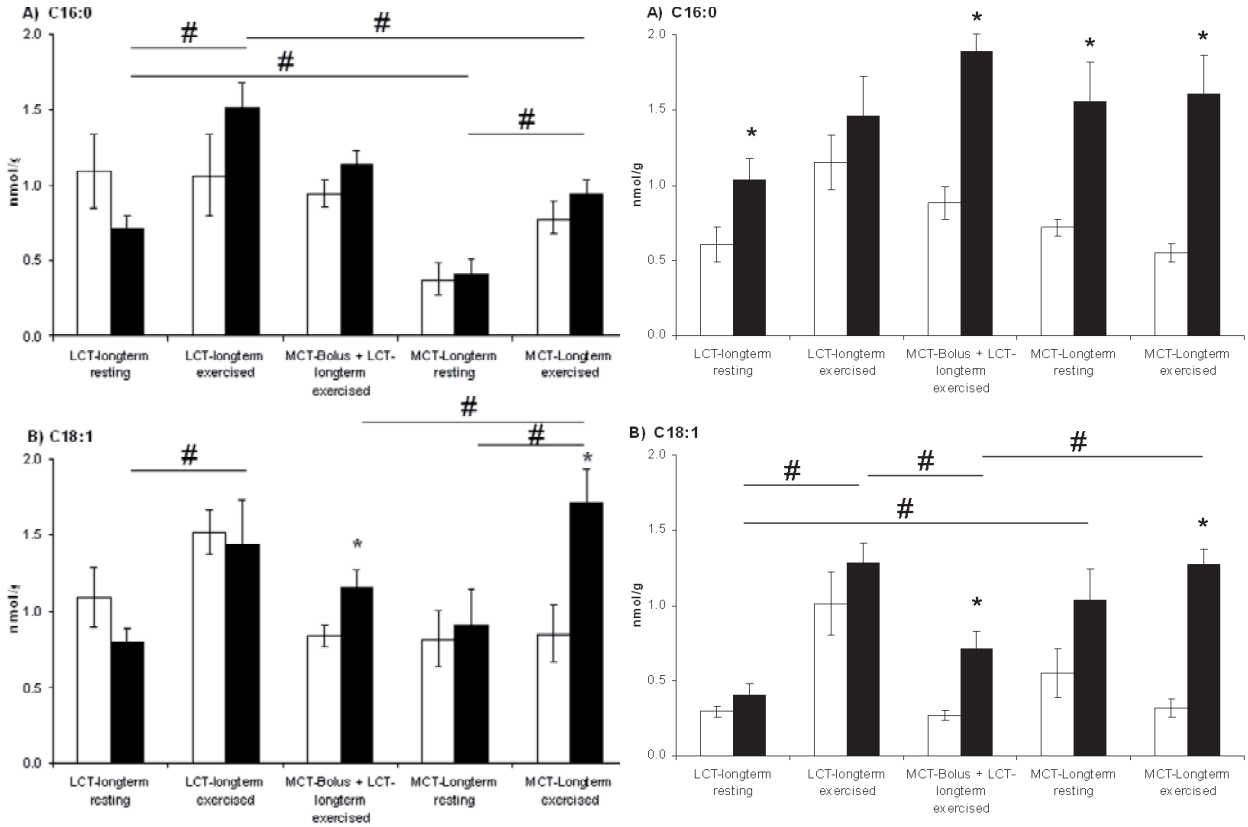


Figure 1: Palmitoyl-carnitine (A) and oleoyl-carnitine (B) concentrations in skeletal muscle of wild-type (WT) (n = 5) and very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice (n = 5). Acylcarnitine concentrations are presented in nmol/g wet weight of five differently treated groups. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± standard error of the mean (SEM). *p < 0.05 indicates significant differences between WT and VLCAD KO mice performed by Student´s t test; #p < 0.05 indicates significant differences between VLCAD KO mice in different groups performed by two-way analysis of variance (ANOVA). Samples were analyzed in duplicate.

Acylcarnitine accumulation increased with MCT-diet

Skeletal muscle. In the VLCAD KO mouse model C16:0 (palmitoyl)-carnitine and C18:1 (oleoyl)-carnitine are the predominantly accumulating acylcarnitines after exercise. These long-chain acylcarnitines increased twofold in VLCAD KO mice compared with WT mice after exercise. This was in marked contrast to VLCAD KO mice fed the LCT diet with pre-exercise MCT bolus. Here, only palmitoyl-carnitine showed a significant increase compared with WT mice after an identical exercise period, but palmitoyl-carnitine as well as oleoyl-

carnitine was significantly lower than in VLCAD KO mice fed the LCT diet. Oleoyl-carnitine concentrations were significantly increased in rested VLCAD KO mice fed the MCT diet compared with WT mice. After exercise, VLCAD KO mice fed the MCT diet presented very high concentrations of these long-chain acylcarnitines compared with exercised VLCAD KO mice fed the LCT diet and were significantly increased compared with mice on the LCT diet with pre-exercise MCT bolus. VLCAD KO mice fed the MCT diet displayed palmitoyl- and oleoyl-carnitine values of approximately 30 nmol/g wet weight after exercise, whereas mice fed the LCT diet accumulated only up to 20 nmol/g wet weight after exercise and mice fed the LCT diet with pre-exercise MCT bolus only up to 10 nmol/g wet weight (Figure 1).



Supplements: Palmitoyl-carnitine (A) and oleoyl-carnitine (B) concentrations in liver (1) and blood (2) of wild-type (WT) (n = 5) and very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice (n = 5). Acylcarnitine concentrations are presented in nmol/g wet weight (1) and µmol/L (2) of five differently treated groups. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± standard error of the mean (SEM). *$p < 0.05$ indicates significant differences between WT and VLCAD KO mice performed by Student´s t test; #$p < 0.05$ indicates significant differences between VLCAD KO mice in different groups performed by two-way analysis of variance (ANOVA). Samples were analyzed in duplicate.

Liver and blood. Liver and blood long-chain acylcarnitines values were between 0.5 and 2 µmol/L lower than skeletal muscle acylcarnitines. There was no significant difference between WT and VLCAD KO mice for palmitoyl-carnitine in the liver. Significant changes in the liver between rested and exercised mice or between mice fed the MCT diet and the LCT diet of VLCAD KO mice were always within the physiological range of WT mice (data added as Supplement 1A). MCT treatment and exercise significantly increased oleoyl-carnitine concentrations in the VLCAD KO mice after MCT bolus as well as MCT long-term treatment (data added as Supplement 1B). In blood, palmitoyl-carnitine concentrations were significantly increased in VLCAD KO mice compared with WT mice in all observed groups except exercised mice treated with LCT (data added as Supplement 2A). Oleoyl-carnitine concentrations were increased comparable with liver (data added as Supplement 2B).

Free carnitine concentration changes with MCT diet

Independent of the type of diet, free carnitine concentrations in skeletal muscle significantly decreased in exercised VLCAD KO mice compared with exercised WT mice (Figure 2 A). In the liver, free carnitine concentrations significantly increased after exercise in VLCAD KO mice compared with WT mice (Figure 2 B).

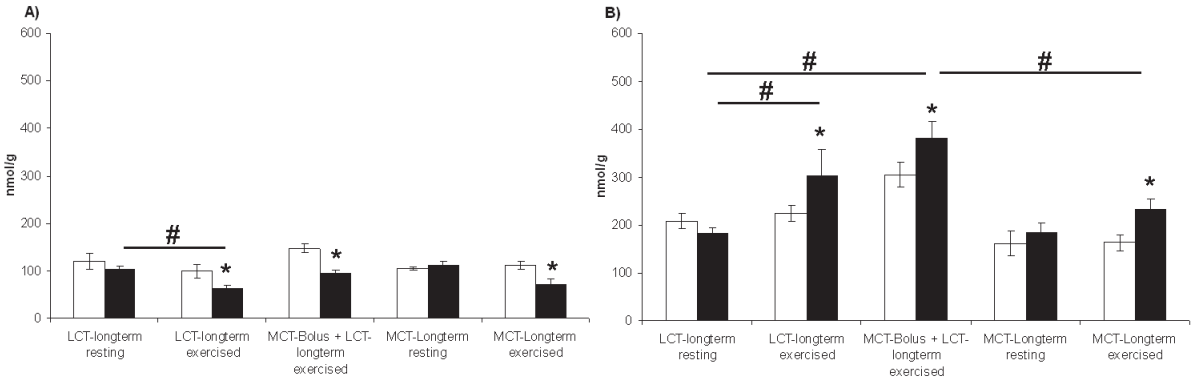


Figure 2: Free carnitine concentrations in skeletal muscle (A) and liver (B). Mean free carnitine concentrations in nmol/g wet weight are shown in wild-type (WT) (n = 5) and very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice (n = 5) after receiving different dietary regimens combined with exercise. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± standard error of the mean (SEM). *p < 0.05 indicates significant differences between WT and VLCAD KO mice performed by Student´s t test; #p < 0.05 indicates significant differences between VLCAD KO mice in different groups performed by two-way analysis of variance (ANOVA). Samples were analyzed in duplicate.

High C8 (octanoyl)-carnitine concentrations after MCT bolus application

In skeletal muscle an almost twofold increase of octanoyl-carnitine concentration was found in both genotypes after receiving the LCT diet with pre-exercise MCT bolus compared with mice on long-term LCT and MCT diets after exercise. Mice fed the MCT diet displayed no significant increase in octanoyl-carnitine concentrations in skeletal muscle following exercise compared with mice fed the LCT diet following exercise (Figure 3). In the liver, no significant changes of octanoyl-carnitine concentrations were observed in the different mouse groups (data not shown).

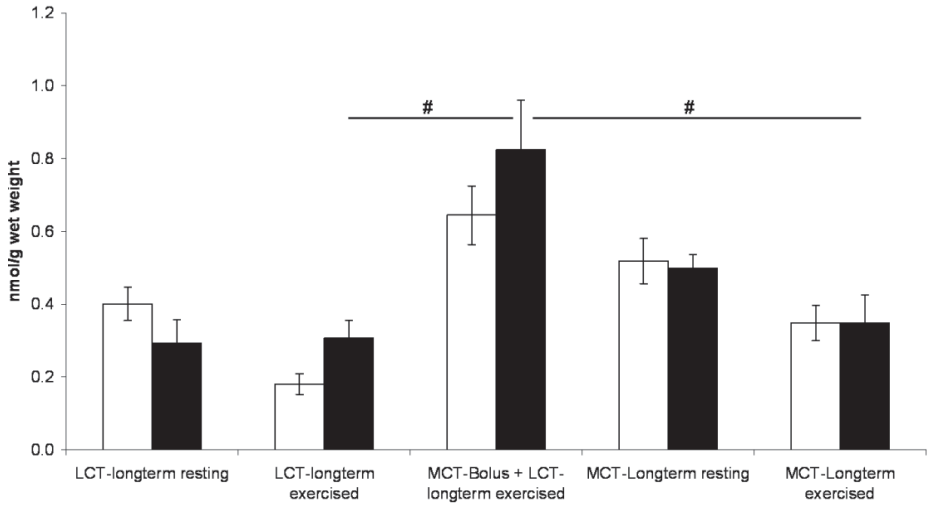


Figure 3: Octanoyl-carnitine concentrations in skeletal muscle. Mean free carnitine concentrations in skeletal muscle (nmol/g wet weight) are shown in wild-type (WT) (n = 5) and very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice (n = 5) after receiving different dietary regimens combined with exercise. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± standard error of the mean (SEM). *$p < 0.05$ indicates significant differences between WT and VLCAD KO mice performed by Student´s t test; #$p < 0.05$ indicates significant differences between VLCAD KO mice in different groups performed by two-way analysis of variance (ANOVA). Samples were analyzed in duplicate.

Intrahepatic lipid content increased with MCT diet

Neutral lipid accumulation in liver slices of WT and VLCAD KO mice fed the LCT diet and the MCT diet under resting conditions are displayed in Figure 4. Surprisingly long-term MCT treatment resulted in an enormous increase of the size of hepatic lipid droplets in VLCAD KO mice. A slight increase of the size of hepatic lipid droplets was also detected in WT mice with long-term MCT treatment compared with LCT-fed mice.

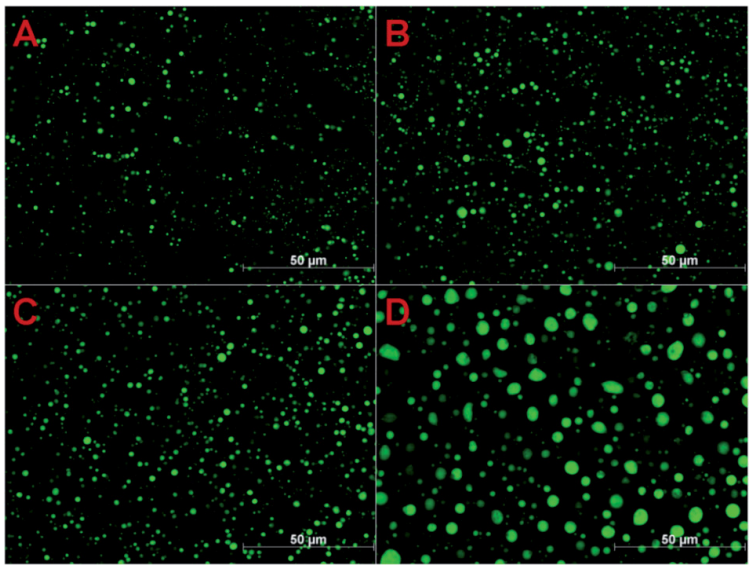


Figure 4: Diet-induced hepatic lipid accumulation. Nile red staining was performed on liver slices of wild-type (WT) mice fed with the long-chain triglycerides (LCT) diet (A), very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice fed with the LCT diet (B), WT mice fed with the medium-chain triglyceride (MCT) diet (C) and VLCAD KO mice fed with the MCT diet (D) after receiving the diet for 5 weeks. A 63×/1.4 objective lens was used for representative fluorescent photomicro-graphs. All mice were analyzed under resting conditions.

Acyl-CoA oxidation capacity changes in the tissues

After exercise, oxidation capacity of palmitoyl-CoA in muscle increased significantly in VLCAD KO mice fed the LCT diet as well as in mice fed the LCT diet with pre-exercise MCT bolus compared with LCT resting mice. In contrast, palmitoyl-CoA oxidation capacity was only slightly, but not significantly, increased in KO mice after exercise receiving the MCT diet (Figure 5A). Using octanoyl-CoA as substrate, oxidation capacity significantly increased after exercise in skeletal muscle of VLCAD KO mice receiving any MCT treatment (Figure 5B).

Oxidation capacity of palmitoyl-CoA in the liver was decreased in resting and exercised VLCAD KO mice compared with WT mice. Interestingly, the exercised VLCAD KO mice and exercised WT mice, presented higher hepatic residual palmitoyl-CoA oxidation capacity with the MCT diet compared with exercised mice with the LCT diet and the LCT diet with pre-exercise MCT bolus (Figure 5C). Exercised mice and mice fed the MCT diet had a significantly lower octanoyl-CoA oxidation capacity in the liver without differences between genotypes compared with resting LCT mice (Figure 5D).

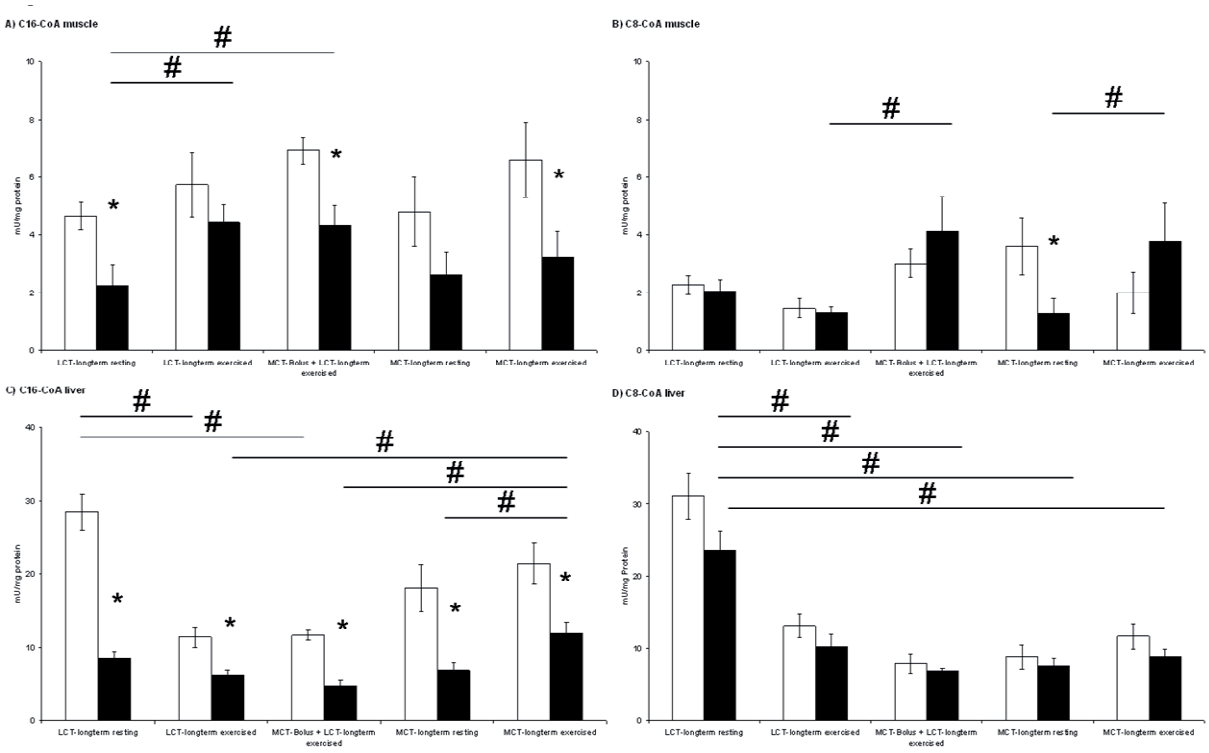


Figure 5: Acyl-CoA dehydrogenase activity (mU/mg protein) with palmitoyl (C16) -CoA and octanoyl (C8) -CoA as substrates are shown in skeletal muscle (A, B) and liver (C, D) of wild-type (WT) (n = 5) and very-long-chain acyl-CoA dehydrogenase (VLCAD) knock-out (KO) mice (n = 5) after receiving different dietary regimens combined with exercise. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± standard error of the mean (SEM). *$p < 0.05$ indicates significant differences between WT and VLCAD KO mice performed by Student´s t test; #$p < 0.05$ indicates significant differences between VLCAD KO mice in different groups performed by two-way analysis of variance (ANOVA). Samples were analyzed in duplicate.

Discussion

Currently, patients with VLCAD deficiency are treated with a fat-reduced and fat-modified diet containing large amounts of MCT to provide sufficient energy and avoid catabolism, tissue accumulation of long-chain fatty acids and their possibly toxic effects (Spiekerkoetter et al. 2009). However, evidence-based treatment recommendations in VLCAD-deficiency are lacking (Vockley et al. 2002). In particular, dietary long-chain fat is exchanged for medium-chain fat to a different extent depending on the severity of the clinical phenotype. It has been hypothesized that MCT is directly available for β-oxidation, but this has never been studied *in vivo,* and the dose and best time of application have never been clearly defined. For example, should it be given throughout the day or only during times of

high energy demand? Our study demonstrates that in order to be beneficial in the murine model, dietary modification with MCT in long-chain fatty acid oxidation disorders has to be clearly adapted to the state of energy demand to be directly oxidized instead of being stored as long-chain fat.

The VLCAD KO mice showed contrasting effects on acylcarnitine concentrations in skeletal muscle after exercise with different MCT applications. Spiekerkoetter et al. (Spiekerkoetter et al. 2005) showed a close correlation between acylcarnitine accumulation and myopathic symptoms. Whereas exercise resulted in significant acylcarnitine accumulation in skeletal muscle of VLCAD KO mice with usual LCT treatment, the application of 2 g/kg body weight of MCT half an hour prior to exercise markedly reduced long-chain acylcarnitine accumulation. MCT is rapidly taken up into the circulatory system and transported as MCFA via the portal vein to skeletal muscles where they are preferentially oxidized (Bach and Babayan 1982; Odle 1997; Benevenga et al. 1989). The observed twofold increase of octanoylcarnitine concentration in skeletal muscle and in addition a corresponding increase of octanoyl-CoA oxidation capacity supports the assumption of rapid MCT uptake and subsequent mitochondrial fatty acid oxidation. Also ketone body production may play a major role with MCT supplementation, as has previously been shown in rats (Shinohara et al. 2005). VLCAD KO mice present low glucose and glycogen concentrations after exercise (Spiekerkoetter et al. 2006). These findings support the hypothesis that ketone body production is required, independent of the supplied diet.

The metabolic situation in mice fed an MCT diet long-term seems to be completely different. Here, MCT are also presumed to be rapidly absorbed and oxidized within hours of consumption. However, our data demonstrate that continuous consumption of MCT throughout the day does not supply the required energy from MCT during increased energy demand. It is rather demonstrated here that a long-term MCT-based diet results in even higher long-chain acylcarnitine concentrations after exercise compared with the regular LCT diet. It has been reported that excess MCT, in contrast to LCT, cannot be stored in adipose tissue (Odle 1997). Moreover, consumption of an MCT-based diet, results in increased hepatic lipogenesis as a result of increased *de novo* fatty acid biosynthesis and increased fatty acid elongation (Carnielli et al. 1994; Crozier 1988; Hill et al. 1990). This is presumably the case with continuous MCT treatment in times of low energy demand, supported by increased lipid storage in mice fed the MCT diet. Also, a decreased hepatic acyl-CoA oxidation capacity in resting MCT-fed mice could be a hint, that β-oxidation is decreased to perform lipogenesis to store the lipids. The liver is an organ that provides metabolites for the production of energy,

rather than providing energy itself. It has also been shown that in human fibroblasts, MCFA first undergo elongation before β-oxidation (Jones et al. 2006). Our findings of increased hepatic lipid accumulation induced by a long-term MCT-based diet also suggests that MCFA are elongated and stored in the liver, as reported previously (Carnielli et al. 1994; Crozier 1988; Hill et al. 1990; Kritchevsky and Tepper 1965; Leveille et al. 1967). In times of increased energy demand, such as during exercise, mainly elongated long-chain fatty acids are then available as energy substrates but cannot be sufficiently metabolized in VLCAD deficiency, subsequently resulting in even higher long-chain acylcarnitine accumulation in skeletal muscle.

After exercise, free carnitine concentrations were always significantly lower in skeletal muscle of VLCAD KO mice. This is most likely due to increased demand of carnitine by the acylcarnitine shuttle through the mitochondrial membranes (Kiens and Roepstorff 2003). Here we demonstrate that a low free-carnitine concentration was also observed in skeletal muscle of VLCAD KO mice on the MCT diet after exercise. We assume that in this diet also, carnitine is required to transport long-chain acylcarnitines through the mitochondrial membranes and is lost by excretion via urine and bile (Bennett et al. 2000; Rinaldo 2001; Hoppel 2003).

In the VLCAD KO mouse, residual acyl-CoA dehydrogenase oxidation capacity with palmitoyl-CoA as substrate occurs due to the presence of enzymes with similar overlapping substrate specificity (Cox et al. 2001). A higher oxidation capacity, as we could demonstrate during exercise in skeletal muscle, suggests that β-oxidation is induced for energy production (McClelland 2004). Provision of MCT as energy source leads to increased oxidation capacity with octanoyl-CoA as substrate suggesting that MCT are also further shortened during exercise in skeletal muscle and therefore have not all been elongated under resting conditions in the liver.

Significantly enhanced long-chain acylcarnitine accumulation in skeletal muscle was observed after exercise in mice on the MCT diet due to impaired fatty acid oxidation. Exercise did not induce hepatic fatty acid oxidation as is supported by reduced oxidation capacity of palmitoyl- and octanoyl-CoA and low hepatic acylcarnitine concentrations. This was in accordance with previous studies reporting no hepatic acylcarnitine accumulation after exercise in mice on a regular LCT diet (Spiekerkoetter et al. 2005).

As with many animal models, the question remains as to whether data in mice can be directly translated to humans. Further prospective studies in humans are essential, especially studies on chronic MCT treatment. However, studies based on tissues, as presented here, are

difficult to perform in humans. Both VLCAD-deficient patients and VLCAD KO mice display similar clinical phenotypes in catabolic situations, despite relatively high residual activities, due to enzymes with overlapping substrate activities (Schuler and Wood 2002; Spiekerkoetter et al. 2005). Since the beginning of newborn screening programs the majority of patients identified with VLCAD deficiency remain asymptomatic until they undergo catabolic situations. Similar stress-induced phenotypes are observed in VLCAD-deficient mice (Liebig et al. 2006; Schymik et al. 2006; Spiekerkoetter et al. 2004; Spiekerkoetter et al. 2005).

In summary, the murine model for VLCAD deficiency shows significantly different effects of a long-term MCT-based diet and an MCT bolus prior to exercise. In case of VLCAD deficiency, continuous MCT feeding leads to significantly increased acylcarnitine production in skeletal muscle, especially after exercise, and increased liver lipid storage. In contrast, additional MCT application prior to exercise improves the metabolic situation. Metabolizable fatty acids are supplied during the exact time frame of increased energy demand and can undergo fatty acid oxidation. This study demonstrates that dietary modification with MCT in long-chain fatty acid oxidation disorders has to be clearly adapted to the state of energy demand to be beneficial and help to prevent myopathy.

Acknowledgments

The study was financially supported by grants from the Deutsche Forschungsgemeinschaft (DFG, SP1125/1-1, SFB 575, SFB 612) and from the Forschungskommission of the Medical Faculty of Heinrich-Heine-University Duesseldorf. We thank Dr. M.D. Laryea, Department of General Pediatrics, Heinrich-Heine-University, Duesseldorf, Germany for expert advice on liquid-chromatography.

References

1. Andresen BS, Olpin S, Poorthuis BJ, Scholte HR, Vianey-Saban C, Wanders R, Ijlst L, Morris A, Pourfarzam M, Bartlett K, Baumgartner ER, deKlerk JB, Schroeder LD, Corydon TJ, Lund H, Winter V, Bross P, Bolund L, Gregersen N (1999) Clear correlation of genotype with disease phenotype in very-long-chain acyl-CoA dehydrogenase deficiency. Am J Hum Genet 64:479-494

2. Bach AC, Babayan VK (1982) Medium-chain triglycerides: an update. Am J Clin Nutr 36:950-962

3. Benevenga NJ, Steinman-Goldsworthy JK, Crenshaw TD, Odle J (1989) Utilization of medium-chain triglycerides by neonatal piglets: I. Effects on milk consumption and body fuel utilization. J Anim Sci 67:3331-3339

4. Bennett MJ, Rinaldo P, Strauss AW (2000) Inborn errors of mitochondrial fatty acid oxidation. Crit Rev Clin Lab Sci 37:1-44

5. Carnielli VP, Sulkers EJ, Moretti C, Wattimena JL, van Goudoever JB, Degenhart HJ, Zacchello F, Sauer PJ (1994) Conversion of octanoic acid into long-chain saturated fatty acids in premature infants fed a formula containing medium-chain triglycerides. Metabolism 43:1287-1292

6. Chegary M, Brinke HT, Ruiter JP, Wijburg FA, Stoll MS, Minkler PE, van WM, Schulz H, Hoppel CL, Wanders RJ, Houten SM (2009) Mitochondrial long chain fatty acid beta-oxidation in man and mouse. Biochim Biophys Acta 1791:806-815

7. Cox KB, Hamm DA, Millington DS, Matern D, Vockley J, Rinaldo P, Pinkert CA, Rhead WJ, Lindsey JR, Wood PA (2001) Gestational, pathologic and biochemical differences between very long-chain acyl-CoA dehydrogenase deficiency and long-chain acyl-CoA dehydrogenase deficiency in the mouse. Hum Mol Genet 10:2069-2077

8. Crozier GL (1988) Medium-chain triglyceride feeding over the long term: the metabolic fate of [14C]octanoate and [14C]oleate in isolated rat hepatocytes. J Nutr 118:297-304

9. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

10. Fowler SD, Greenspan P (1985) Application of Nile red, a fluorescent hydrophobic probe, for the detection of neutral lipid deposits in tissue sections: comparison with oil red O. J Histochem Cytochem 33:833-836

11. Gillingham MB, Scott B, Elliott D, Harding CO (2006) Metabolic control during exercise with and without medium-chain triglycerides (MCT) in children with long-chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD) or trifunctional protein (TFP) deficiency. Mol Genet Metab 89:58-63

12. Greenspan P, Mayer EP, Fowler SD (1985) Nile red: a selective fluorescent stain for intracellular lipid droplets. J Cell Biol 100:965-973

13. Gregersen N, Andresen BS, Corydon MJ, Corydon TJ, Olsen RK, Bolund L, Bross P (2001) Mutation analysis in mitochondrial fatty acid oxidation defects: Exemplified by acyl-CoA dehydrogenase deficiencies, with special focus on genotype-phenotype relationship. Hum Mutat 18:169-189

14. Gregersen N, Bross P, Andresen BS (2004) Genetic defects in fatty acid beta-oxidation and acyl-CoA dehydrogenases. Molecular pathogenesis and genotype-phenotype relationships. Eur J Biochem 271:470-482

15. Hill JO, Peters JC, Swift LL, Yang D, Sharp T, Abumrad N, Greene HL (1990) Changes in blood lipids during six days of overfeeding with medium or long chain triglycerides. J Lipid Res 31:407-416

16. Hoppel C (2003) The role of carnitine in normal and altered fatty acid metabolism. Am J Kidney Dis 41:S4-12

17. Jones PM, Butt Y, Messmer B, Boriak R, Bennett MJ (2006) Medium-chain fatty acids undergo elongation before beta-oxidation in fibroblasts. Biochem Biophys Res Commun 346:193-197

18. Kiens B, Roepstorff C (2003) Utilization of long-chain fatty acids in human skeletal muscle during exercise. Acta Physiol Scand 178:391-396

19. Kritchevsky D, Tepper SA (1965) INFLUENCE OF MEDIUM-CHAIN TRIGLYCERIDE (MCT) ON CHOLESTEROL METABOLISM IN RATS. J Nutr 86:67-72

20. Leveille GA, Pardini RS, Tillotson JA (1967) Influence of medium-chain triglycerides on lipid metabolism in the rat. Lipids 2:287-294

21. Liebig M, Schymik I, Mueller M, Wendel U, Mayatepek E, Ruiter J, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Neonatal screening for very long-chain acyl-coA dehydrogenase deficiency: enzymatic and molecular evaluation of neonates with elevated C14:1-carnitine levels. Pediatrics 118:1065-1069

22. McClelland GB (2004) Fat to the fire: the regulation of lipid oxidation with exercise and environmental stress. Comp Biochem Physiol B Biochem Mol Biol 139:443-460

23. Odle J (1997) New insights into the utilization of medium-chain triglycerides by the neonate: observations from a piglet model. J Nutr 127:1061-1067

24. Papaioannou VE, Johnson RS (1993) Gene targeting: a practical approach. Oxford University Press, Oxford107-146

25. Primassin S, Ter VF, Mayatepek E, Spiekerkoetter U (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. Pediatr Res 63:632-637

26. Rinaldo P (2001) Fatty acid transport and mitochondrial oxidation disorders. Semin Liver Dis 21:489-500

27. Schuler AM, Wood PA (2002) Mouse models for disorders of mitochondrial fatty acid beta-oxidation. ILAR J 43:57-65

28. Schymik I, Liebig M, Mueller M, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Pitfalls of neonatal screening for very-long-chain acyl-CoA dehydrogenase deficiency using tandem mass spectrometry. J Pediatr 149:128-130

29. Shinohara H, Ogawa A, Kasai M, Aoyama T (2005) Effect of randomly interesterified triacylglycerols containing medium- and long-chain fatty acids on energy expenditure and hepatic fatty acid metabolism in rats. Biosci Biotechnol Biochem 69:1811-1818

30. Spiekerkoetter U (2007) Effects of a fat load and exercise on asymptomatic VLCAD deficiency. J Inherit Metab Dis 30:405

31. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de KH, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis 32:498-505

32. Spiekerkoetter U, Ruiter J, Tokunaga C, Wendel U, Mayatepek E, Wijburg FA, Strauss AW, Wanders RJ (2006) Evidence for impaired gluconeogenesis in very long-chain acyl-CoA dehydrogenase-deficient mice. Horm Metab Res 38:625-630

33. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

34. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van VN, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

35. Ter VF, Primassin S, Hoffmann L, Mayatepek E, Spiekerkoetter U (2009) Corresponding increase in long-chain acyl-CoA and acylcarnitine after exercise in muscle from VLCAD mice. J Lipid Res 50:1556-1562

36. Vockley J, Singh RH, Whiteman DA (2002) Diagnosis and management of defects of mitochondrial beta-oxidation. Curr Opin Clin Nutr Metab Care 5:601-609

37. Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, Ijlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. J Inherit Metab Dis 22:442-487

Chapter 6

Medium-chain triglycerides impair lipid metabolism and induce hepatic steatosis in very long-chain acyl-CoA dehydrogenase (VLCAD)-deficient mice

Sara Tucci, Sonja Primassin, Frank ter Veld and Ute Spiekerkoetter

Molecular Genetics and Metabolism, 2010 Jun; accepted for publication
(DOI:10.1016/j.ymgme.2010.05.005)

Abstract

A medium-chain-triglyceride (MCT)-based diet is mainstay of treatment in very-long chain acyl-CoA dehydrogenase deficiency (VLCADD), a long-chain fatty acid β-oxidation defect. Beneficial effects have been reported with an MCT-bolus prior to exercise. Little is known about the impact of a long-term MCT-diet on hepatic lipid metabolism. Here we investigate the effects of MCT-supplementation on liver and blood lipids in the murine model of VLCADD.

Wild-type (WT) and VLCAD-knock-out (KO) mice were fed 1) a long-chain triglyceride (LCT)-diet over 5 weeks, 2) an MCT-diet over 5 weeks and 3) an LCT-diet plus MCT-bolus. Blood and liver lipid content were determined. Expression of genes regulating lipogenesis was analyzed by RT-PCR.

Under the LCT-diet, VLCAD-KO mice accumulated significantly higher blood cholesterol concentrations compared to WT mice. The MCT-diet induced severe hepatic steatosis, significantly higher serum free fatty acids and impaired hepatic lipid mobilization in VLCAD-KO mice. Expression at mRNA level of hepatic lipogenic genes was up-regulated.

The long-term MCT diet stimulates lipogenesis and impairs hepatic lipid metabolism in VLCAD-KO mice. These results suggest a critical reconsideration of a long-term MCT-modified diet in human VLCADD. In contrast, MCT in situations of increased energy demand appears to be a safer treatment alternative.

Introduction

Mitochondrial β-oxidation of fatty acids is an important process for provision of energy necessary for a wide variety of metabolic reactions. Therefore, its dysfunctions, e.g. due to inherited enzyme defects may have severe consequences on human health with involvement of different organ systems. Deficiency of the very long-chain acyl-CoA dehydrogenase (VLCAD) is the most common β-oxidation disorder of long-chain fatty acids with an incidence of 1:55.000 to 1:100.000 births (Spiekerkoetter et al. 2003). The clinical phenotype of VLCAD deficiency is very heterogeneous and presents with different severity and age of onset (Spiekerkoetter et al. 2003). Before newborn screening for fatty acid oxidation disorders was implemented, the most severe phenotype became manifest in the first weeks and months of life with cardiomyopathy and hepatopathy. The infantile hepatic phenotype presented with hypoketotic hypoglycemia and lethargy and was triggered by preceding infections. The milder later-onset myopathic phenotype became usually manifest in adolescence or young adulthood with muscle weakness, rhabdomyolysis and myoglobinuria (Gregersen et al. 2001; Kompare and Rizzo 2008). Overall, fasting and other catabolic stress as results of illnesses or prolonged exercise may induce or aggravate clinical symptoms and under these conditions severe metabolic derangement may occur.

VLCAD-deficient patients are unable to oxidize fatty acids originating from the diet or endogenous lipolysis, which subsequently accumulate as long-chain acyl-CoAs and acyl-carnitines (Spiekerkoetter et al. 2004). So far, treatment recommendations for VLCAD deficiency include the avoidance of fasting and a fat-modified and long-chain triglyceride (LCT)-restricted diet (Spiekerkoetter et al. 2009b). Fat modification includes the replacement of LCT by medium-chain triglycerides (MCT). In fact, it is generally accepted that, medium-chain fatty acids can cross the mitochondrial membrane as carboxylates without previous esterification into acylcarnitines by carnitine palmitoyl-CoA transferases (CPT I) and can be fully metabolized (Bach and Babayan 1982; Spiekerkoetter et al. 2009b).

A rather novel dietary approach with MCT has been reported to have great beneficial effects during periods of increased energy demand. In this case, MCT is administered as bolus immediately prior to exercise in patients with VLCAD deficiency, carnitine palmitoyl-CoA transferase II (CPT-II) deficiency and long chain hydroxy acyl-CoA-dehydrogenase (LCHAD) deficiency (Bonnefont et al. 2004; Gillingham et al. 2006; Spiekerkoetter 2007). Despite the positive reports of MCT treatment to maintain metabolic control in a number of patients with different long-chain fatty acid oxidation defects, there have also been scattered

reports that point to adverse effects of MCT-supplementation on liver function of patients with intractable epilepsy (Beverley and Arthur 1988). Therefore, the actual impact on lipid homeostasis and lipid clearance, especially in VLCAD deficiency, remains to be tested.

To study the effects of dietary interventions, the VLCAD knock-out (KO) mouse represents an excellent animal model as it displays a very similar clinical phenotype to human VLCAD deficiency (Spiekerkoetter et al. 2004). For instance, in the absence of stress VLCAD KO mice appear asymptomatic but triggers such as fasting, cold exposure and physical exercise give rise to accumulation of long-chain acylcarnitines (e.g. C16:0 and C18:1) and result in hypoglycaemia, hepatopathy and skeletal myopathy (Spiekerkoetter et al. 2005; Spiekerkoetter et al. 2006).

To address the question how MCT affects lipid homeostasis and lipid clearance, we studied wild-type (WT) and VLCAD KO mice fed with a long-term MCT-diet and an MCT-bolus treatment, in an effort to compare dietary effects with a normal LCT diet. To gain insight into fat metabolism under different stress conditions, mice were submitted to different workloads. We analyzed liver and blood lipid concentrations at rest, after physical exercise, and after 24 hours of regeneration following exercise. Furthermore, we analyzed the effects of the MCT-based diet on the expression of genes regulating *de novo* biosynthesis and elongation of fatty acids. Our results clearly demonstrate that the long-term MCT diet in VLCAD KO mice has a profound impact on lipid mobilization and clearance.

Materials and Methods

Reagents

All chemicals used were purchased in p.a. quality from J.T. Backer (Griesheim, Germany), Merck (Darmstadt, Germany), Riedel de Haën (Seelze, Germany), Roche (Penzberg, Germany), and Sigma-Aldrich (Deisenhofen, Germany).

Animals

VLCAD KO mice used in these studies were kindly provided by A.W. Strauss (currently Cincinnati Children's Hospital, Ohio, USA) and were generated as described in detail previously (Exil et al. 2003). Experiments were performed on fourth- to fifth-generation intercrosses of C57BL6+129sv VLCAD genotypes. Littermates served as controls and genotyping of mice was performed as described previously (Exil et al. 2003).

Groups consisting of five mice aged 10-12 week-old were analyzed 1) under well-fed, resting conditions, 2) after short-term exercise of 1 hour and 3) after 24 h regeneration following exercise. Blood samples were collected by heart puncture and serum was obtained by centrifugation at 16,000 *g* for 10 min and stored at -80 °C for further analysis. The mice were either sacrificed immediately, after exercise or 24 h after the exercise protocol was terminated. Liver was rapidly removed and immediately frozen in liquid nitrogen.

All animal studies were performed with the approval of the Heinrich-Heine-University Institutional Animal Care and Use Committee. The care of the animals was in accordance with the Heinrich-Heine-University Medical Center and Institutional Animal Care and Use Committee guidelines.

Diet composition and supplementation

After weaning, at approx. 5-7 weeks of age, mice were divided in three groups and fed with different diets for 5 weeks. The first group received a purified mouse diet containing 5.1 % crude fat in form of LCT, corresponding to 13 % of metabolizable energy calculated with Atwater factors (ssniff® EF R/M Control, ssniff Spezialdiäten GmbH, Soest, Germany). The second group was fed with a diet (ssniff® EF R/M control, ssniff GmbH, Soest, Germany) corresponding, as well, to ca. 13 % of total metabolizable energy calculated with Atwater factors, in which 4.4 % from a total of 5 % fat, were MCT- (Ceres®MCT-oil, basis GmbH, Oberpfaffenhofen, Germany) while the remaining 0.6 % derived from the essential soy bean oil. In both diets the carbohydrate and protein contents were unmodified and corresponded to 65 % and 22 % of metabolizable energy, respectively. A third group received long term the same LCT diet as group 1 but was supplemented in addition half an hour prior to exercise an oral MCT-oil bolus of 2 g/kg body weight.

Exercise protocol

As mice are nocturnal animals treadmill running was performed during the dark cycle. Ten to twelve weeks old WT and VLCAD KO mice were exercised 60 min on a Columbus Instruments Simplex II metabolic rodent treadmill consisting of four individual lanes without inclination and a shock plate incentive (3 Hz, 200 msec, 160 V, 1.5 mA). The training protocol was performed as already described by ter Veld et al. (ter Veld et al. 2008) with minor modifications. Briefly, mice were placed in an exercise chamber, and after an adaptation period of 15 min, initial belt speed was set to 4 m/min and increased every 5 min by 2 m/min to a maximum of 16 m/min. Mice ran until they displayed signs of exhaustion (> 2 s spent on the shocker plate without attempting to re-engage the treadmill).

Lipid and lipoprotein analysis

Serum was collected 90 minutes from the last meal. Free fatty acid and lipoprotein concentrations were measured as duplicates in serum samples by using enzymatic kits (free fatty acid quantification kit, Biocat, Heidelberg, Germany; EnzyChrom HDL and VLDL/LDL Assay kit, BioTrend, Cologne, Germany) on an Infinite M200 Tecan (Crailsheim, Germany) plate reader. Serum TGAs and liver TGAs were measured as duplicates by using enzymatic kits (EnzyChrom Triglyceride Assay kit BioTrend Cologne, Germany and Triglyceride Quantification kit, Biocat, Heidelberg, Germany, respectively). All assays were performed following the manufacturer's instructions.

Intrahepatic lipid content

The intrahepatic lipid content was measured gravimetrically. Lipids were extracted according to a method by Folch et al. (FOLCH et al. 1957) modified as follows. Lyophilized liver (20-40 mg) was homogenized in 0.5 mL distilled water and extracted in 4 mL chloroform/methanol ($CHCl_3$/MeOH) 2:1 (v/v) by gentle agitation at 4 °C for 3 h. 0.8 mL of 0.9 % NaCl was added to the mixture, shaken vigorously and centrifuged at 2500 *g* for 10 min. The organic phase containing the extracted lipids was removed and evaporated under nitrogen flow at 40 °C.

RT-PCR analysis

Total liver RNA was isolated with the RNeasy mini kit (Qiagen, Hilden Germany). Forward and reverse primers for β-actin (BC138614), fatty acid synthase (FASN; NM_007988.3), acyl-CoA carboxylase (ACC-1α; NM_133360.2), sterol regulatory element binding transcription factor 1 (SREBP-1c; BC056922.1) and stearoyl-Coenzyme A desaturase (SCD1; NM_009127.4) were designed with the FastPCR program (R. Kalendar, Institute of Biotechnology, Helsinki) and are available on request. RT-PCR was performed in a single step procedure with the QuantiTect SYBR Green^{TM} RT-PCR (Qiagen, Hilden, Germany) on an Applied Biosystems 7900 HT Sequence Detection System in Micro Amp 96-well optical reaction plates capped with MicroAmp optical caps (Applied Biosystems, Foster City, CA, USA) as previously described (Schafer et al. 2007). The values in all samples were normalized to the expression level of the internal standard β-actin.

Statistical analysis

Reported data are presented as means ± standard error of the mean (SEM) with *n* denoting the number of animals tested. Analysis for the significance of differences was performed using Student's t-tests for paired and unpaired data. Two-way analysis of variance (ANOVA)

with Bonferroni post-test was performed using GraphPad Prism (GraphPad Software, San Diego California USA). Differences were considered significant if $p < 0.05$.

Results

VLCAD KO mice were not able to perform completely the exercise protocol under both LCT and MCT diet. In contrast VLCAD KO mice that received the MCT bolus immediately prior to exercise fully managed the exercise protocol without exhaustion, as for the WT mice.

Clinical phenotype

The mean body weights were not significantly different between WT and VLCAD mice in the different groups fed with the LCT or with the MCT diet (data not shown). In contrast, the ratio liver/body weight was higher in VLCAD KO mice under LCT diet than in the WT mice. Under resting conditions both genotypes fed with the long-term MCT diet showed significantly higher ratios when compared to the mice groups with the LCT diet (**Table 1**), suggesting increased hepatic fat accumulation under MCT.

Table 1

Effect of LCT and MCT diet on liver/body weight ratio.

		WT	VLCAD KO
LCT	**resting**	3.9 ±0.1	4.5 ±0.1*
	exercise	4.9 ±0.5	4.9 ±0.3
	regeneration	4.8 ±0.2	4.9 ±0.4
MCT	**resting**	5.0 ±0.3#	5.2 ±0.2#
	exercise	5.0 ±0.4	5.0 ±0.3
	regeneration	4.9 ±0.2	5.4 ±0.2

Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. Significances were calculated by Student´s t-test and two-way ANOVA with Bonferroni post-test. Ratio is mean ± SEM ($n = 5$).

Intrahepatic lipid content

Effects of the LCT diet. WT and VLCAD KO mice fed with the LCT diet long-term did not show intrahepatic lipid accumulation under resting conditions and after exercise. In both

groups the lipid content was significantly reduced after 24 h of regeneration following exercise, as shown in **Fig. 1**.

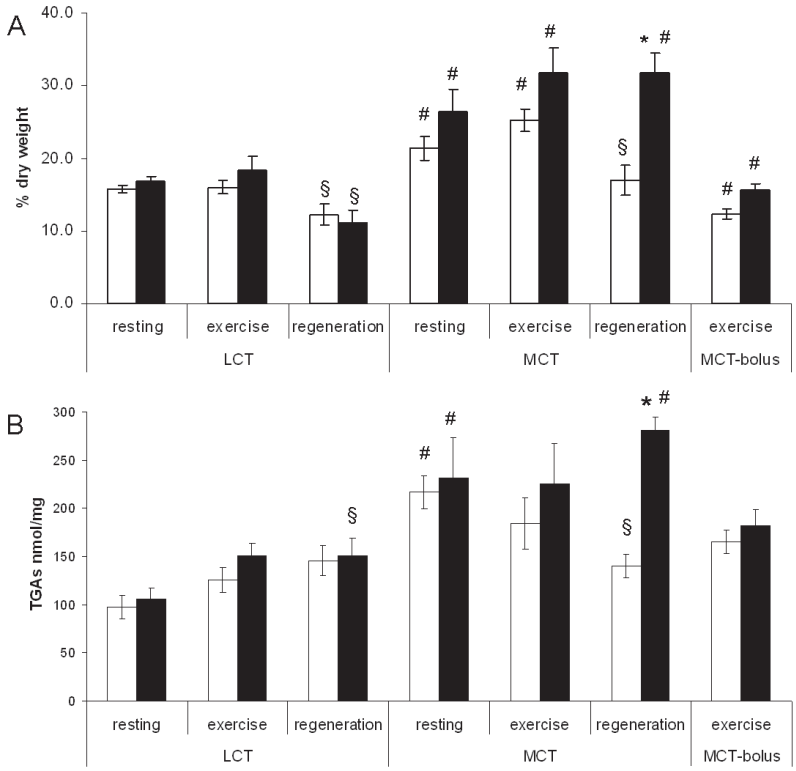


Figure 1: Intrahepatic (A) lipid content and (B) triglyceride (TGA) content. Mean concentrations are expressed in dry weight (% dw) for the lipid content and nmol/mg for TGA content. The values are mean ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice per dietary group under resting conditions, after exercise and after 24 h of regeneration following exercise. White bars and black bars represent WT and VLCAD KO mice, respectively. LCT: long-chain triacylgyceride. MCT: medium-chain triglycerides. Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. § indicates significant differences between WT or VLCAD KO mice under resting, exercised and regenerated condition in the same dietary regimen. Significances were calculated by Student´s t-test and two-way ANOVA with Bonferroni post-test.

Under resting conditions, the liver triglyceride (TGA) content in WT and VLCAD KO mice fed with the LCT diet long-term corresponded to 98.0 ± 12.3 nmol/mg and 106.1 ± 10.8 nmol/mg, respectively (**Fig. 1**). In both groups the TGA concentration was significantly increased after exercise, but showed no further accumulation after 24 h of regeneration.

Effects of the MCT diet. In contrast, long-term MCT treatment resulted in a significant increase in liver lipid content up to ca. 21.4 ± 1.6 % of the dry weight in WT and up to ca. 26.4 ± 3.1 % in VLCAD KO mice (**Fig. 1**). Exercise contributed to a further increase in lipid accumulation in both genotypes. After 24 h of regeneration only the lipid content in WT mice decreased to 17.0 ± 2.1 % of the dry weight, while in VLCAD KO mice it remained high with a value of 31.8 ± 2.8 %.

The MCT diet induced two-fold higher TGA accumulation in liver of WT and VLCAD KO mice with 216.4 ± 17.3 nmol/mg and 231.6 ± 41.9 nmol/mg, respectively. After exercise and 24 h of regeneration in WT mice liver TGAs significantly decreased, whereas in VLCAD KO mice we observed the opposite trend with a further, albeit not significant, TGA accumulation.

Effects of the MCT-bolus. WT and VLCAD KO mice that received the MCT-bolus immediately prior to exercise showed significantly lower ($p<0.01$) liver lipid content when compared to exercised mice fed with the long-term MCT diet (**Fig. 1**). In contrast, application of the MCT-bolus caused a higher, though not significant, increment of liver TGA in both genotypes.

Blood lipid profile

Effects of the LCT diet. Compared to the WT mice, VLCAD KO mice showed no significantly higher concentrations of serum TGAs and free fatty acids (FFA) under resting conditions (**Fig. 2**). After exercise and 24 h of regeneration the levels of the serum lipids displayed a steady increase in both genotypes, however, this was only significant in WT mice, suggesting increased lipid mobilizations.

Importantly, compared to WT, VLCAD KO mice accumulated significantly higher concentrations of total cholesterol at rest and after exercise (**Fig. 3**). However, a further significant increase was observed in both genotypes after 24 h of regeneration with values of 164.4 ± 4.9 mg/dL and 164.1 ± 2.4 mg/dL for the WT mice and the VLCAD KO mice, respectively.

Effects of the MCT diet. Although the difference was not significant ($p>0.05$), the long-term MCT diet raised TGA levels in VLCAD KO mice compared to WT mice under resting conditions (**Fig. 2**). Exercise significantly increased the serum TGAs in both mice groups. After 24 h of regeneration serum TGA concentrations were 329.4 ± 28.4 mg/dL in the WT and 313.1 ± 60.6 mg/dL in the VLCAD KO. In parallel to serum TGA, serum FFA in VLCAD KO was four-fold higher compared to the WT mice. Whereas exercise and regeneration following exercise did not further increase FFA concentration in VLCAD KO

mice, WT mice presented a tremendous increase in FFA concentration after exercise and regeneration (**Fig. 2**), suggesting increased lipolysis as a physiological response in WT mice. VLCAD KO mice constantly display high FFA levels most likely as a sign of significantly impaired further metabolism of long-chain fatty acids deriving from MCT.

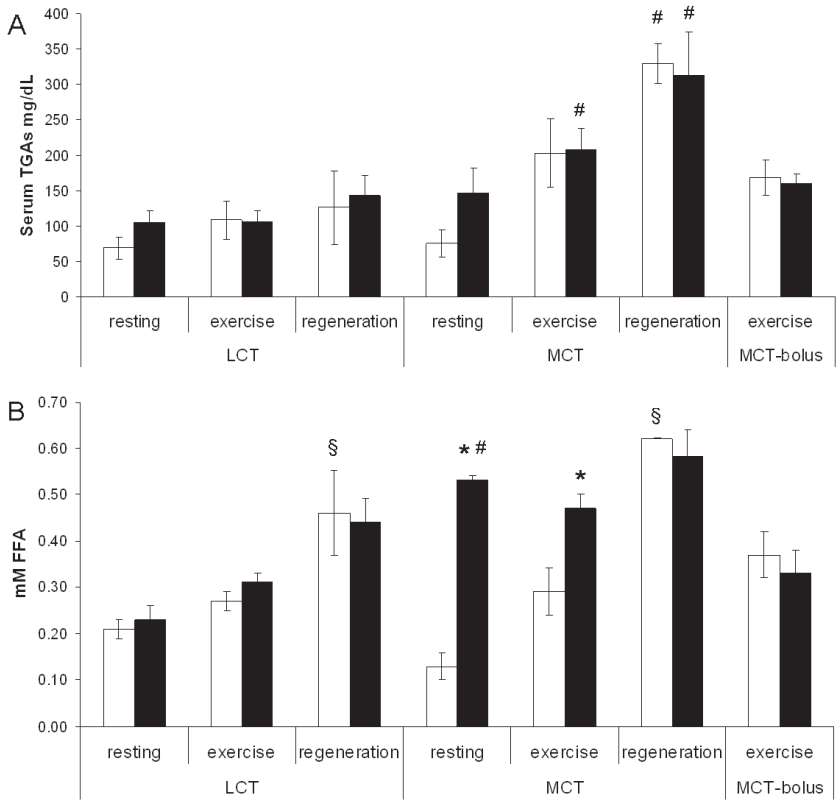


Figure 2: Serum (A) triglycerides (TGA) content and (B) free fatty acid (FFA) content. Mean concentrations are expressed in mg/dL for the TGA and mM for FFA. The values are mean ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice per dietary group under resting conditions, after exercise and after 24 h of regeneration following exercise. White bars and black bars represent WT and VLCAD KO mice, respectively. LCT: long-chain triacylgyceride. MCT: medium-chain triglycerides. Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. § indicates significant differences between WT or VLCAD KO mice under resting, exercised and regenerated condition in the same dietary regimen. Significances were calculated by Student´s t-test and two-way ANOVA with Bonferroni post-test.

Long-term MCT treatment increased total cholesterol in both genotypes under resting conditions (**Fig. 3**). However, the lipoprotein subgroups VLDL/LDL and HDL cholesterol were only significantly increased in the VLCAD KO mice. VLDL/LDL was even two-fold

increased in VLCAD KO mice compared to WT mice with 20.3 ± 2.8 mg/dL and 48.0 ± 2.3 mg/dL, respectively. Exercise and regeneration resulted in a decrease in total cholesterol, VLDL/LDL and HDL cholesterol in VLCAD KO mice. In strong contrast, all three increased in WT mice after exercise, but resulted in significant difference after 24 hours of regeneration with much lower concentrations in VLCAD KO than in WT mice.

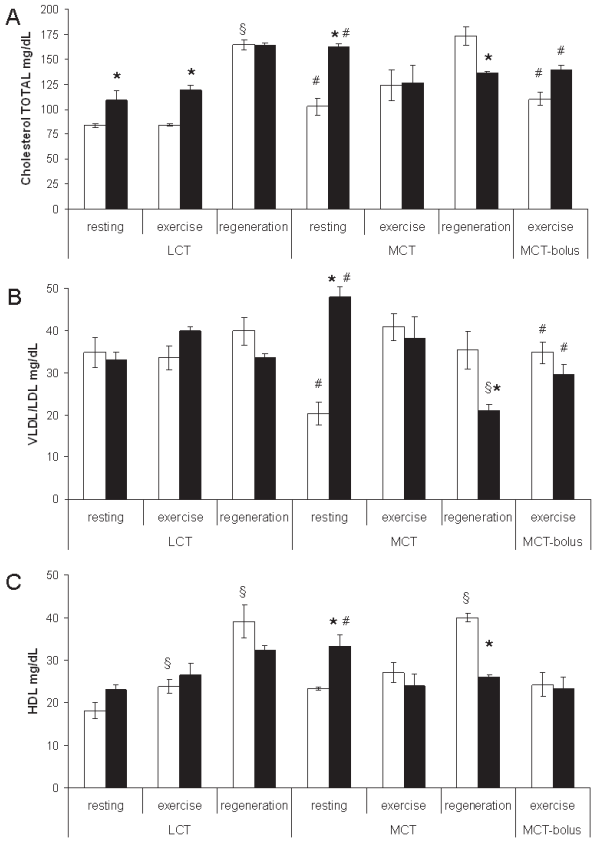


Figure 3: Cholesterol concentration in serum sample of WT and VLCAD KO mice. Graphs represent total serum cholesterol (A), VLDL/LDL cholesterol (B) and HDL cholesterol (C). Mean concentrations are expressed in mg/dL. The values are mean ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice per dietary group under resting conditions, after exercise and after 24 h of regeneration following exercise. White bars and black bars represent WT and VLCAD KO mice, respectively. LCT: long-chain triacylgyceride. MCT: medium-chain triglycerides. Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. § indicates significant differences between WT or VLCAD KO mice under resting, exercised and regenerated condition in the same dietary regimen. Significances were calculated by Student´s t-test and two-way ANOVA with Bonferroni post-test. Samples were analyzed in duplicate.

Effects of the MCT-bolus. The application of an MCT-bolus prior to exercise did not significantly raise serum TGA and FFA levels but rather significantly increased total cholesterol concentrations in both genotypes. VLDL/LDL was significantly reduced in the VLCAD KO mice compared to those mice under long-term LCT and long-term MCT diets, while HDL concentrations were not affected at all (**Fig. 3**).

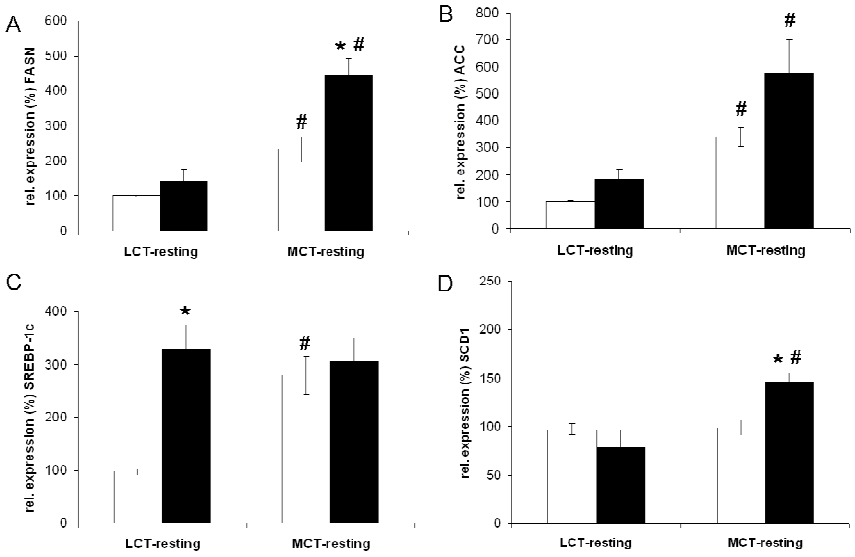


Figure 4: Relative expression of FASN (A), ACC-1α (B), SREBP-1c (C) and SCD1 (D) genes at mRNA level. White bars and black bars represent WT and VLCAD KO mice, respectively. LCT: long-chain triacylglyceride. MCT: medium-chain triglyceride. Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. Significances were calculated by Student´s t-test and two-way ANOVA with Bonferroni post-test. Values are mean of mean ± SEM for WT ($n = 5$) and VLCAD KO ($n = 5$).

Long-term MCT diet results in up-regulation of lipogenic genes

To test how MCT affects hepatic lipogenesis, we analyzed the expression at mRNA level of sterol regulatory element binding transcription factor 1 (SREBP-1c) that directly activate the acetyl-CoA carboxylase (ACC-1α) and the fatty acid synthase (FASN) involved in *de novo* biosynthesis and elongation of short- and medium-chain fatty acids, respectively (**Fig. 5**). Because of the significantly higher serum FFA concentrations we also tested the expression of stearoyl-Coenzyme A desaturase (SCD1), which is regulated by fatty acids and carbohydrates and it is responsible for the biosynthesis of unsaturated fatty acids (Ntambi 1995). RT-PCR revealed that the expression of the lipogenic genes was significantly up-regulated in both genotypes under the MCT diet at rest (**Fig. 4**). Under this condition the

expression at mRNA level in VLCAD KO mice compared to WT mice was two-fold higher for FASN and three-fold higher for the ACC-1α. Interestingly, the expression of SREBP-1c was three-fold higher in VLCAD KO mice under LCT diet compared to the WT mice. Feeding with the long-term MCT diet did not further raise the mRNA expression of SREBP-1c in VLCAD KO mice, whereas we observed a significant up-regulation in WT mice.

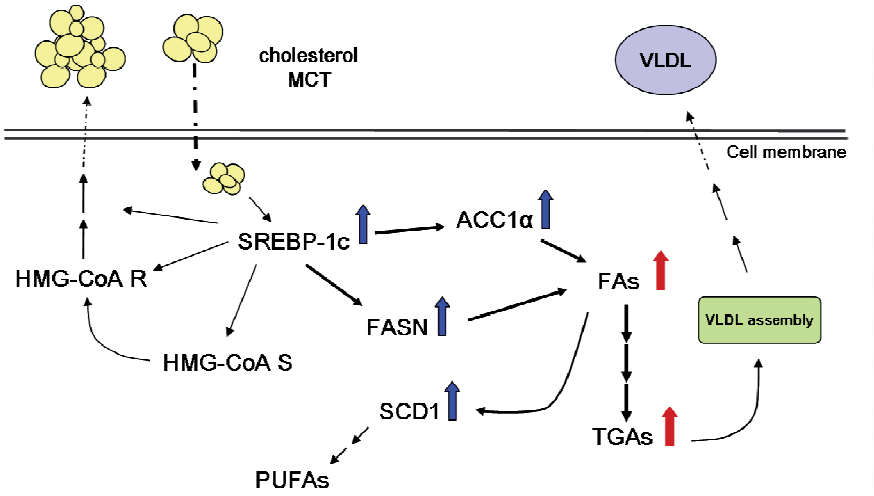


Figure 5: Simplified model of up-regulation of lipogenesis in VLCAD KO mice under MCT diet on the basis of our results.

Sterol regulatory element binding transcription factor 1 (SREBP-1c) directly activate the acetyl-CoA carboxylase (ACC-1α) and the fatty acid synthase (FASN) involved in *de novo* biosynthesis and elongation of short- and medium-chain fatty acids, respectively, as previously described (Horton et al. 1998). Fatty acids (FA) are converted to triglycerides (TGA) and at the same time they regulate the expression of stearoyl-CoA desaturase (SCD1; Ntambi et al. 1996). MCT: medium-chain triglyceride; PUFAs: polyunsaturated fatty acids; HMG-CoA S: 3-hxdroxy-3-methylglutaryl-CoA synthase; HMG-CoA R: 3-hxdroxy-3-methylglutaryl-CoA reductase.

Discussion

In this work, we demonstrate that replacement of LCT with MCT, without increasing total dietary fats, as is common practice in the treatment of symptomatic VLCAD deficiency, results in severe hepatic steatosis and impaired fat metabolism. Moreover, we observed a strong up-regulation of SREBP-1c, FASN, ACC-1α and SCD1 genes suggesting that the

elongation and *de novo* biosynthesis of fatty acids exceeds the breakdown of medium-chain fatty acids. In contrast, the application of additional MCT prior intensive physical exercise in mice with an LCT-diet appears not to cause steatosis and impaired lipid metabolism, despite an overall increase in fat intake.

MCT but not LCT increases liver lipid and TGA content.

We could not observe any differences in liver lipid accumulation between WT and VLCAD KO mice at rest when fed with the LCT diet. In addition, the strong reduction in liver lipids after a regeneration period of 24 hours demonstrates a normal physiological response to exercise in both genotypes. In fact, fatty acids that are released by adipocytes are transported to the liver, where they are, together with liver lipids, re-esterified and subsequently secreted to meet situations of higher energy demand (Klein et al. 1989). The increment in TGA concentration after exercise and regeneration confirms that in both genotypes fed with the LCT diet the ability of the liver to mobilize the needed lipids is not impaired.

In strong contrast, the long-term MCT diet results in severe hepatic steatosis and two-fold higher accumulation of TGA in both genotypes under resting conditions, in line with a very recent study (Primassin et al. 2010). However, whereas WT mice are able to mobilize stored hepatic fatty acids during physical exercise and subsequent regeneration, reducing hepatic lipid content, we no longer observe this reduction in VLCAD KO liver upon regeneration, but rather observe a further increase in liver TGA (**Fig. 1**). These findings clearly suggest that in VLCAD KO mice the long-term MCT diet results in impaired fat metabolism whereby the liver is no longer capable to process, re-ester and secrete lipids. As an additional and detrimental effect, our study reveals that MCT treatment results in an increased hepatic lipogenesis. This can already be demonstrated in WT mice and is even much more obvious in VLCAD KO mice (Fig. 4). MCT treatment induces a nearly four-fold higher expression at mRNA level of FASN and three-fold increment in the expression of ACC-1α in VLCAD KO mice compared to the LCT diet, as resumed in Fig. 5. Of note, previous studies are indeed supportive of this MCT-induced effect on lipogenesis (Carnielli et al. 1994; Crozier 1988). The expression of SREBP-1c, fundamentally involved in lipogenesis and cholesterol homeostasis and regulated directly by cholesterol, is already significantly increased in VLCAD KO mice under the LCT-diet but it does not result in hepatic lipid accumulation. However, the regulation of lipogenesis and cholesterol biosynthesis is an extremely complex system of signals that all together contribute to activation or inhibition of the different pathways. Many other factors not analyzed in this study may play a crucial role in the up-

regulation of SREBP-1c leading to the biosynthesis of cholesterol but not on the stimulation of lipogenesis, as occurred in VLCAD KO mice under LCT diet.

Long-term MCT diet alters blood lipid composition and impairs VLDL/LDL secretion

Lipid homeostasis is regulated by the balance between hepatic lipogenesis and hepatic lipid utilization and mobilization, as well as blood lipid clearance. The analysis of postprandial serum in mice receiving an LCT treatment shows that the concentration of serum FFA and TGA correlated indeed with liver TGA levels, thus reflecting the decrease in liver lipid content.

TGA mobilization is particularly evident in exercised and regenerated mice fed with the MCT diet. Whereas in WT mice this increment of serum TGA is paralleled by a simultaneous decrease in liver TGA, this no longer occurs in VLCAD KO mice. These results are again clearly indicative of seriously impaired fat metabolism and cannot only be ascribed to defective fatty acid oxidation itself.

Liver FFA influx is mainly a concentration-dependent mechanism, as the hepatic uptake of fatty acids is not regulated (Lavoie and Gauthier 2006). Considering the very high intrahepatic lipid amounts observed in VLCAD KO mice fed with the MCT diet at rest, a significantly higher amount of serum FFAs is not surprising. In line with this observation, Guo *et al.* (Guo et al. 2006) demonstrated that octanoate inhibits lipogenesis in adipocytes and thereby induces a fasting-like condition, which helps to explain the very high serum FFA levels in VLCAD KO mice fed with the MCT diet.

Since VLCAD KO mice are not able to metabolize long-chain free fatty acids, these are converted into TGA and thereby potentially lipotoxic circulatory FFA is neutralized (Donnelly et al. 2005; Unger et al. 1999).

For the first time, we show that LCT-fed VLCAD KO mice accumulate, both at rest as well as after exercise, significant amounts of total cholesterol very probably to ascribe to an increase of esterified cholesterol as the concentrations of VLDL/LDL and HDL were not significantly higher to those detected in WT mice. In contrast, MCT treatment itself causes significantly higher serum cholesterol in both genotypes as well, in line with results obtained in humans (Cater et al. 1997; Tholstrup et al. 2004) suggesting a careful monitoring of the complete lipid status in patients with fatty acid oxidation defects independent of the applied diet.

Despite the positive lipoprotein turnover observed in both genotypes after prolonged exercise and after the period of regeneration under the LCT diet in VLCAD KO mice we note a marked reduction in serum VLDL/LDL levels, supporting the hypothesis that MCT also

impairs secretion of serum lipoproteins. Very recently it has been reported that the inhibition of mitochondrial β-oxidation reduces the efficiency of VLDL production (Yamaguchi et al. 2007) and an impaired synthesis and/or secretion of lipoproteins from the liver was put forward as reason for the pathogenesis of non-alcoholic steatohepatitis (Fujita et al. 2009). In VLCAD deficient patients elevated transaminases have also been observed during metabolic derangement and stress-induced fatty liver possibly suggesting similar effects (Schuler and Wood 2002).

MCT-bolus does not affect liver and serum lipids

Here we demonstrate that additional MCT in a situation of increased energy demand does not induce hepatic steatosis and TGA accumulation in the liver of WT and VLCAD KO mice. Clinically, the application of a MCT-bolus immediately prior to prolonged exercise has already been reported to be successful in patients to prevent episodic muscular pain or rhabdomyolysis induced by increased energy demand (Bonnefont et al. 2004; Gillingham et al. 2006; Spiekerkoetter 2007). These data also support, that additional MCT in VLCAD deficient patients should be individually adapted to meet the need during increased energy demand. Moreover, in this study we did not analyze repeated application of MCT-bolus for longer time periods, therefore currently we cannot evaluate the long-term effects of the bolus therapy on lipid metabolism.

Conclusions

In conclusion, this study has yielded strong evidence that the MCT-based diet has a profound impact on lipid metabolism and clearance in VLCAD KO mice, resulting in excessive accumulation of liver TGA and, consequently, severe hepatic steatosis. We postulate that all the following factors: engineered ablation of VLCAD and impaired long-chain fat oxidation, up-regulation of lipogenesis, reduced ability to secrete VLDL/LDL lipoproteins and, finally, enhanced hepatic fatty acid influx, act in concert and represent the underlying pathophysiological mechanism for development of serious liver disease. Thus, this unforeseen strong impairment of fat metabolism in VLCAD KO mice suggests that long-term MCT application as therapy for human VLCAD deficiency at least calls for critical reconsideration. Although our findings were obtained in the mouse model of VLCAD deficiency, which can obviously never allow full extrapolation to the VLCAD deficient patient, however, the clinical phenotypes in both are very similar in many respects (Fujita et al. 2009; Spiekerkoetter et al. 2005). It is, therefore, conceivable and valid, to suspect that comparable effects as described here may appear in VLCAD deficient patients. However, the amount of MCT in the mouse diet used in this work does not correspond to the recommended

dietary restrictions in asymptomatic newborn with VLCAD deficiency, as the ratio LCT to MCT is about 50:50 (Arnold et al. 2009; Spiekerkoetter et al. 2009a). Nevertheless, in most of the commercial available infant formulas LCT are nearly completely replaced by MCT with the exception of essential long-chain fatty acids, in line with our study.

Likewise, on the basis of the significant higher serum cholesterol levels observed in VLCAD KO mice under LCT diet, we call for caution and suggest careful monitoring of lipid status in VLCAD deficient patients. Further studies to characterize the exact pathogenic mechanisms responsible for impaired lipid metabolism in fatty acid oxidation defects under MCT treatment are currently under way.

The application of a single MCT-bolus immediately prior to intensive and prolonged exercise, however, appears to be a more safe dietary measure that provides the required energy during increased energy turnover and neither impairs lipid metabolism nor results in hepatic steatosis.

Acknowledgements

The study was financially supported by grants from the Deutsche Forschungsgemeinschaft (DFG SP1125/1-1, SFB 575 and SFB 612) and from the Forschungskommission of the Medical Faculty of Heinrich-Heine-University Düsseldorf. We thank Dr. L. Hoffmann for advice in RT-PCR analysis.

References

1. Arnold GL, Van Hove J, Freedenberg D, Strauss A, Longo N, Burton B, Garganta C, Ficicioglu C, Cederbaum S, Harding C, Boles RG, Matern D, Chakraborty P, Feigenbaum A (2009) A Delphi clinical practice protocol for the management of very long chain acyl-CoA dehydrogenase deficiency. Mol Genet Metab 96:85-90

2. Bach AC, Babayan VK (1982) Medium-chain triglycerides: an update. Am J Clin Nutr 36:950-962

3. Beverley D, Arthur R (1988) Fatty liver and medium chain triglyceride (MCT) diet. Arch Dis Child 63:840-842

4. Bonnefont JP, Djouadi F, Prip-Buus C, Gobin S, Munnich A, Bastin J (2004) Carnitine palmitoyltransferases 1 and 2: biochemical, molecular and medical aspects. Mol Aspects Med 25:495-520

5. Carnielli VP, Sulkers EJ, Moretti C, Wattimena JL, van Goudoever JB, Degenhart HJ, Zacchello F, Sauer PJ (1994) Conversion of octanoic acid into long-chain saturated fatty acids in premature infants fed a formula containing medium-chain triglycerides. Metabolism 43:1287-1292

6. Cater NB, Heller HJ, Denke MA (1997) Comparison of the effects of medium-chain triacylglycerols, palm oil, and high oleic acid sunflower oil on plasma triacylglycerol fatty acids and lipid and lipoprotein concentrations in humans. Am J Clin Nutr 65:41-45

7. Crozier GL (1988) Medium-chain triglyceride feeding over the long term: the metabolic fate of [14C]octanoate and [14C]oleate in isolated rat hepatocytes. J Nutr 118:297-304

8. Donnelly KL, Smith CI, Schwarzenberg SJ, Jessurun J, Boldt MD, Parks EJ (2005) Sources of fatty acids stored in liver and secreted via lipoproteins in patients with nonalcoholic fatty liver disease. J Clin Invest 115:1343-1351

9. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

10. FOLCH J, LEES M, SLOANE STANLEY GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509

11. Fujita K, Nozaki Y, Wada K, Yoneda M, Fujimoto Y, Fujitake M, Endo H, Takahashi H, Inamori M, Kobayashi N, Kirikoshi H, Kubota K, Saito S, Nakajima A (2009) Dysfunctional very-low-density lipoprotein synthesis and release is a key factor in nonalcoholic steatohepatitis pathogenesis. Hepatology 50:772-780

12. Gillingham MB, Scott B, Elliott D, Harding CO (2006) Metabolic control during exercise with and without medium-chain triglycerides (MCT) in children with long-chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD) or trifunctional protein (TFP) deficiency. Mol Genet Metab 89:58-63

13. Gregersen N, Andresen BS, Corydon MJ, Corydon TJ, Olsen RK, Bolund L, Bross P (2001) Mutation analysis in mitochondrial fatty acid oxidation defects: Exemplified by acyl-CoA dehydrogenase deficiencies, with special focus on genotype-phenotype relationship. Hum Mutat 18:169-189

14. Guo W, Xie W, Han J (2006) Modulation of adipocyte lipogenesis by octanoate: involvement of reactive oxygen species. Nutr Metab (Lond) 3:30

15. Horton JD, Shimomura I, Brown MS, Hammer RE, Goldstein JL, Shimano H (1998) Activation of cholesterol synthesis in preference to fatty acid synthesis in liver and adipose tissue of transgenic mice overproducing sterol regulatory element-binding protein-2. J Clin Invest 101:2331-2339

16. Klein S, Peters EJ, Holland OB, Wolfe RR (1989) Effect of short- and long-term beta-adrenergic blockade on lipolysis during fasting in humans. Am J Physiol 257:E65-E73

17. Kompare M, Rizzo WB (2008) Mitochondrial fatty-acid oxidation disorders. Semin Pediatr Neurol 15:140-149

18. Lavoie JM, Gauthier MS (2006) Regulation of fat metabolism in the liver: link to non-alcoholic hepatic steatosis and impact of physical exercise. Cell Mol Life Sci 63:1393-1409

19. Ntambi JM (1995) The regulation of stearoyl-CoA desaturase (SCD). Prog Lipid Res 34:139-150

20. Ntambi JM, Sessler AM, Takova T (1996) A model cell line to study regulation of stearoyl-CoA desaturase gene 1 expression by insulin and polyunsaturated fatty acids. Biochem Biophys Res Commun 220:990-995

21. Primassin S, Tucci S, Herebian D, Seibt A, Hoffmann L, Ter VF, Spiekerkoetter U (2010) Pre-exercise medium-chain triglyceride application prevents acylcarnitine accumulation in skeletal muscle from very-long-chain acyl-CoA-dehydrogenase-deficient mice. J Inherit Metab Dis

22. Schafer C, Hoffmann L, Heldt K, Lornejad-Schafer MR, Brauers G, Gehrmann T, Garrow TA, Haussinger D, Mayatepek E, Schwahn BC, Schliess F (2007) Osmotic regulation of betaine homocysteine-S-methyltransferase expression in H4IIE rat hepatoma cells. Am J Physiol Gastrointest Liver Physiol 292:G1089-G1098

23. Schuler AM, Wood PA (2002) Mouse models for disorders of mitochondrial fatty acid beta-oxidation. ILAR J 43:57-65

24. Spiekerkoetter U (2007) Effects of a fat load and exercise on asymptomatic VLCAD deficiency. J Inherit Metab Dis 30:405

25. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de Klerk H, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009a) Management and outcome in 75 individuals with long-chain fatty acid oxidation defects: results from a workshop. J Inherit Metab Dis 32:488-497

26. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de KH, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009b) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis

27. Spiekerkoetter U, Ruiter J, Tokunaga C, Wendel U, Mayatepek E, Wijburg FA, Strauss AW, Wanders RJ (2006) Evidence for impaired gluconeogenesis in very long-chain acyl-CoA dehydrogenase-deficient mice. Horm Metab Res 38:625-630

28. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U (2003) MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr 143:335-342

29. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

30. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van Vlies N, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

31. ter Veld F, Primassin S, Hoffmann L, Mayatepek E, Spiekerkoetter U (2008) Corresponding increase in long-chain acyl-CoA and acylcarnitine after exercise in muscle from very long-chain acyl-CoA dehydrogenase-deficient mice. J Lipid Res

32. Tholstrup T, Ehnholm C, Jauhiainen M, Petersen M, Hoy CE, Lund P, Sandstrom B (2004) Effects of medium-chain fatty acids and oleic acid on blood lipids, lipoproteins, glucose, insulin, and lipid transfer protein activities. Am J Clin Nutr 79:564-569

33. Unger RH, Zhou YT, Orci L (1999) Regulation of fatty acid homeostasis in cells: novel role of leptin. Proc Natl Acad Sci U S A 96:2327-2332

34. Yamaguchi K, Yang L, McCall S, Huang J, Yu XX, Pandey SK, Bhanot S, Monia BP, Li YX, Diehl AM (2007) Inhibiting triglyceride synthesis improves hepatic steatosis but exacerbates liver damage and fibrosis in obese mice with nonalcoholic steatohepatitis. Hepatology 45:1366-1374

Chapter 7

Effects of various fat-modified diets in very long-chain acyl-CoA dehydrogenase deficient mice

Sonja Primassin, Sara Tucci and Ute Spiekerkoetter

Abstract

Background. Very long-chain acyl-CoA dehydrogenase (VLCAD) deficiency is the most common long-chain fatty acid oxidation defect presenting with heterogeneous clinical phenotypes. Some patients remain asymptomatic. Disease triggers are situations of increased energy demand when energy production predominantly relies on fat metabolism. Long-chain fat restriction and fat modification are current treatment approaches. However, it is under debate how much fat should be restricted and whether a fat overload may worsen the clinical phenotype.

Methods. VLCAD knock-out (KO) mice and wild-type (WT) littermates were fed with a fat-rich diet (fat = 24 % of energy), a Western diet (42 % of energy) or a fat-reduced diet (7% of energy) for 5 weeks and compared with VLCAD KO and WT mice fed with a regular mouse diet (13 % of energy). Long-chain acylcarnitines, free carnitine and γ-butyrobetaine concentrations were determined in liver and skeletal muscle at rest and after one hour exercise. Intrahepatic lipid and triglyceride (TGA) content were quantified during the different conditions.

Results. VLCAD KO mice neither developed a clinical phenotype under both fat-rich diets nor under the fat-reduced diet. However, VLCAD KO mice were not able to conclude the one hour running protocol under all diets. At the same time, long-chain acylcarnitines significantly accumulated in skeletal muscle of VLCAD KO mice after exercise, with exception of the Western diet. Intrahepatic lipid content was significantly higher in VLCAD KO mice after exercise under both fat-rich diets and the fat-reduced diet compared with the regular diet. Triglyceride content was exceptionally high after exercise in VLCAD KO mice fed with the fat-reduced diet.

Conclusions. A fat-reduced diet did not prevent myopathic symptoms in VLCAD KO mice after exercise. Sufficient energy supply appears to be essential to avoid myopathy as demonstrated for the Western diet, a high-caloric diet. In addition, a fat-restricted diet did not prevent hepatic lipid accumulation after exercise due to fat release from adipocytes.

Introduction

Mitochondrial β-oxidation defects are inherited in an autosomal recessive manner. Very long-chain acyl-coenzyme A dehydrogenase (VLCAD) is one of the enzymes initializing the mitochondrial β-oxidation and is responsible for the first oxidation step of long-chain fatty acids. Deficiency of VLCAD leads to a number of different symptoms including cardiomyopathy, hepatic encephalopathy, hepatic steatosis and rhabdomyolysis under variable catabolic stress situations with an occurrence of approximately 1:50.000 to 1:100.000 births (Gregersen et al. 2004; Spiekerkoetter et al. 2003). Because of clinical heterogeneity, environmental factors are widely debated.

Newborn screening (NBS) for many disorders of mitochondrial β-oxidation including VLCAD deficiency is performed in several countries worldwide. Presymptomatic identification can prevent catastrophic events in affected individuals, especially sudden death (Liebig et al. 2006; Schulze-Bergkamen et al. 2005; Schymik et al. 2006; Vockley et al. 2002) and has significantly reduced disease morbidity and mortality.

Diverse treatment approaches are currently being evaluated concerning dietary fat intake and frequency of meals (Arnold et al. 2009). Nevertheless, fat-restriction and fat-modification have been reported to be important treatment interventions (Spiekerkoetter et al. 2009) and various studies have proven their effectiveness (Gillingham et al. 2006; Spiekerkoetter 2007). However, only recently, fat restriction has been loosened for some milder phenotypes of VLCAD deficiency. Supplementation with medium-chain triglycerides (MCT) has been effective to prevent or reverse cardiomyopathy and skeletal myopathy (Brown-Harrison et al. 1996; Roe et al. 2002). Hepatopathy, in contrast, did not improve with MCT. In fact, hepatic steatosis even increased with a long-term MCT-enriched diet (Primassin et al. 2010; Tucci et al. 2010).

One important question concerning dietary treatment is how much LCT should be allowed and what effects does an LCT overload have on liver metabolism and carnitine homeostasis. “Secondary carnitine deficiency” occurs in VLCAD deficiency and carnitine depletion is still widely debated to be an important pathogenetic factor in fatty acid oxidation defects. In the VLCAD-deficient mouse, however, stimulation of endogenous carnitine biosynthesis has been shown to restore low carnitine tissue levels without the need of supplemented carnitine (Primassin et al. 2008; Vaz and Wanders 2002).

Free carnitine and acylcarnitines in blood serve as monitoring parameters in patients and indicate long-term metabolic control. γ-butyrobetaine levels reflect the activity of carnitine biosynthesis in tissues (Primassin et al. 2008).

We here studied the effects of two fat-enriched diets and one fat-restricted diet on liver and skeletal muscles in the VLCAD deficient mouse at rest and after defined exercise.

Materials and methods

Animals

VLCAD KO mice were generated as previously described (Exil et al. 2003; Papaioannou VE and Johnson RS 1993; Spiekerkoetter et al. 2005) and were provided by Prof. Dr. Arnold Strauss, former Vanderbilt University Hospital, Nashville, TN, USA, now University of Cincinnati, College of Medicine, Cincinnati, OH, USA. Genotypes were determined by duplicate polymerase chain reaction (PCR) analysis and analysis was performed as previously described (Spiekerkoetter et al. 2005). Experiments were performed on fourth- to fifth-generation intercrosses of C57BL6+129sv VLCAD genotypes. Littermates served as controls. At the age of 10-12 weeks mice of both genotypes were analyzed under well-fed, resting conditions and after short-term exercise. Animals were divided into four different treatment groups, each receiving a diet with a different amount of fat. Each group consisted of five mice (n = 5). All mice were weighed and sacrificed by cervical dislocation, the mice in the exercised group immediately after termination of exercise. Liver and skeletal muscle samples were rapidly removed and frozen immediately in liquid nitrogen. All animal studies were performed with the approval of the Heinrich-Heine-University Institutional Animal Care and Use Committee. Care of the animals was in accordance with the Heinrich-Heine-University Medical Centre and Institutional Animal Care and Use Committee guidelines.

Diet composition

After being weaned, the first group of wild-type (WT) and VLCAD KO mice received a defined mouse diet containing 5.1 % crude fat (ssniff® EF R/M Control, ssniff Spezialdiäten GmbH), corresponding to 13 % of metabolizable energy calculated with Atwater factors. This diet is subsequently referred to as control diet. The second group of WT and VLCAD KO mice received a diet containing 10.6 % crude fat (ssniff® EF R/M control, ssniff GmbH). The amount of fat has been doubled in comparison with the control diet and corresponds to 24 % of total metabolizable energy calculated with Atwater factors. The protein content remained unchanged, but the starch content was reduced. This diet is subsequently referred to as fat-rich diet. The third group of WT and VLCAD KO mice was fed on a diet containing 21.2 % crude fat (ssniff® EF R/M from TD88137 mod., ssniff GmbH), corresponding to 42 % of metabolizable energy calculated with Atwater factors. The amount of fat has been quadrupled

in comparison with the control diet. Starch and protein content has been reduced, therefore sugar content has been tripled. This diet is subsequently referred to as western type diet. The fourth group of WT and VLCAD KO mice was fed on a diet containing 2.6 % crude fat (ssniff® EF R/M control, ssniff GmbH), corresponding to 7 % of metabolizable energy calculated with Atwater factors. The fat content has been halved compared with the control diet. The protein content remained unchanged and starch content was increased. This diet is subsequently referred to as fat-reduced diet (Table 1).

Table 1: Nutrient content of diets. Metabolizable energy (ME) is shown in percentage (%) of fat, carbohydrates and protein (A) and crude nutrients (B) are shown in % for the four applied diets.

A

ME [%]	control	fat-rich	western	fat-reduced
Fat	13	24	42	7
Carbohydrates	65	55	43	70
Protein	22	21	15	23
ME [MJ/kg]	15.4	16.7	22.1	15

B

crude nutrients [%]	control	fat-rich	western	fat-reduced
crude fat	5.1	10.6	21.2	2.6
crude protein	20.7	20.8	17.1	20.9
starch	46.5	41.4	14.5	49.2
sugar	11.7	11.6	32.8	11.6

Exercise protocol

As mice are nocturnal animals treadmill running was performed during the dark cycle. Three-month-old WT and VLCAD KO animals were exercised 60 min on a Columbus Instruments Simplex II metabolic rodent treadmill consisting of four individual lanes without inclination and an electric shock grid (10 mAmp, frequency of 10 Hz). Mice were placed in an exercise chamber; after an adaptation period of 15 min initial belt speed was set to 4 m/min and increased every 5 min by 2 m/min to a maximum of 16 m/min. Mice were exercised until they displayed signs of exhaustion or the exercise was terminated after 60 min. Exhaustion was defined as resting more than 15 $s*min^{-1}$ on the electric shock grid or as falling back on the electric shock grid more than 15 $times*min^{-1}$ (Spiekerkoetter et al. 2004).

Analysis of carnitine and acylcarnitines

Analysis of free carnitine (C0), γ-butyrobetaine and acylcarnitines was performed in skeletal muscle and liver as previously described (Primassin et al. 2008). Briefly, C0, γ-butyrobetaine and acylcarnitines were extracted from liver and skeletal muscle in the presence of internal standards ([$^2H_3$] carnitine, for C0 and γ-butyrobetaine concentrations; [$^2H_3$]C16 carnitine, for C14-C18 carnitines) with acetonitrile/water (ACN/H_2O) (80/20 % v/v). The extracted tissues were dried and finally C0, γ-butyrobetaine and acylcarnitines were analyzed by ESI-MS/MS as their butyl esters. C0, γ-butyrobetaine and all even-chain C14-C18 acylcarnitines (saturated and unsaturated) were measured. Internal standards were applied according to van Vlies et al. (van Vlies et al. 2005) to the tissues.

Intrahepatic lipid and TGA content

The intrahepatic lipid content was measured gravimetrically. Lipids were extracted according to a method by Folch et al. (FOLCH et al. 1957) and modified as follows. Lyophilized liver (20-40 mg) was homogenized in 0.5 ml distilled water and extracted in 4 ml chloroform/methanol ($CHCl_3$/MeOH) 2:1 (v/v) by gentle agitation at 4 °C for 3 h. 0.8 ml of 0.9 % NaCl was added to the mixture, shaken vigorously and centrifuged at 2500 *g* for 10 min. The organic phase containing the extracted lipids was removed and evaporated under nitrogen flow at 40 °C. Liver TGAs were measured as duplicates by using an enzymatic kit (Triglyceride Quantification kit, Biocat, Heidelberg, Germany). The assay was performed following the manufacturer's instructions.

Statistical analysis

Data were acquired and analyzed using MassLynx NT v4.0 (Micromass, UK). Data are presented as the means ± standard error of the mean (SEM). Statistical analysis of differences between two means was assessed by Student's *t* test. Multiple means were compared by a two-way analysis of the variance (ANOVA) with a Bonferroni post hoc test (GraphPad Prism 4·0, San Diego, CA, USA). A probability level of $p < 0.05$ was regarded as significant.

Results and discussion

Clinical phenotype

In this study we demonstrate that in the VLCAD KO mouse model two high-fat diets and a low-fat diet applied for a period of 5 weeks were not associated with clinical symptoms. Male and female mice were equally mixed among the groups. Body weights were not

significantly different between mice of both sexes at the age of 10-12 weeks and not significantly different in VLCAD KO mice compared with WT littermates (Table 2). Importantly, also the different diets did not affect body weight in VLCAD KO mice after 5 weeks. However, the diet in humans and mice generally contains different fat amounts. Humans naturally ingest higher amounts of fat (30 % energy from fat) than mice, which only ingest 13 % energy from fat with the recommended regular diet. The body weight remained unchanged in VLCAD KO mice after one hour of exercise under the different dietary conditions (Table 2).

Table 2: Body weight in [g] in VLCAD KO mice and their WT littermates after 5 weeks of treatment under resting and exercised conditions. Values are means ± SEM. Values were not significantly different.

	Condition	*n*	WT	KO
control	Resting	5	24 (± 0.8)	26 (± 2.2)
	Exercised	5	27 (± 2.3)	24 (± 1.6)
fat-rich	Resting	5	25 (± 2.2)	25 (± 2.3)
	Exercised	5	23 (± 2.0)	25 (± 1.3)
western	Resting	5	27 (± 0.6)	24 (± 1.7)
	Exercised	5	25 (± 1.8)	23 (± 1.4)
fat-reduced	Resting	5	27 (± 1.0)	26 (± 1.2)
	Exercised	5	24 (± 1.3)	25 (± 1.4)

All WT mice independent of the dietary group were able to complete the running protocol at 16 m/min over 60 min. VLCAD KO mice were generally exhausted before the 60 min time span was elapsed in all dietary groups. Thus, running time was reduced in VLCAD KO mice compared with WT mice in each group (Table 3). Running times were not significantly different between VLCAD KO mice of the different dietary groups suggesting that a higher fat intake did not induce myopathy. As shown in Table 3, a diet with a low fat content did not improve running capacity either. Myopathy has been shown in VLCAD KO mice to be associated with long-chain acylcarnitine accumulation (Primassin et al. 2008; Spiekerkoetter et al. 2005). In fact, long-chain acylcarnitines significantly increase in skeletal muscle under all dietary conditions with the exception of the Western diet, a high-caloric diet, suggesting that sufficient energy supply appears to be essential to avoid acylcarnitine accumulation.

Table 3: Running time [min] in VLCAD KO mice and their WT littermates after 5 weeks of dietary application. Values are means ± SEM. Values are not significantly different.

	n	WT	KO
control	5	60 (± 0.3)	55 (± 1.2)
fat-rich	5	60 (± 0.0)	54 (± 3.2)
western	5	60 (± 0.5)	58 (± 0.9)
fat-reduced	5	60 (± 0.0)	49 (± 5.1)

Carnitine / acylcarnitine homeostasis in skeletal muscle with different dietary regimens

VLCAD KO mice and WT littermates did not present any significant changes in carnitine, γ-butyrobetaine and acylcarnitine concentrations under different dietary conditions at rest, indicating that carnitine / acylcarnitine homeostasis is not affected by different dietary treatment. After exercise, VLCAD KO mice fed the regular diet or the fat-rich diet displayed significantly lower free carnitine concentrations in skeletal muscle than WT littermates. Interestingly, VLCAD KO mice fed with the fat-reduced or the western type diet did not display decreased free carnitine concentrations after exercise compared to WT mice (Figure 1A). At the same time, γ-butyrobetaine concentrations were significantly increased in skeletal muscle after exercise in VLCAD KO mice fed with the control diet or the fat-rich diet, whereas concentrations remained low in VLCAD KO mice fed with the fat-reduced diet or the western diet (Figure 2A). These results are in line with previous studies demonstrating a negative correlation between free carnitine and γ-butyrobetaine concentrations (Primassin et al. 2008). Low free carnitine concentrations reflect accumulation of long-chain acylcarnitines and indeed, acylcarnitines in skeletal muscle only mildly increased with the western type diet compared with the normo-caloric fat-rich diet (Figure 3A) suggesting that an increased energy supply may prevent acylcarnitine accumulation and carnitine deficiency. However, this hypothesis is not completely true for the fat-reduced diet.

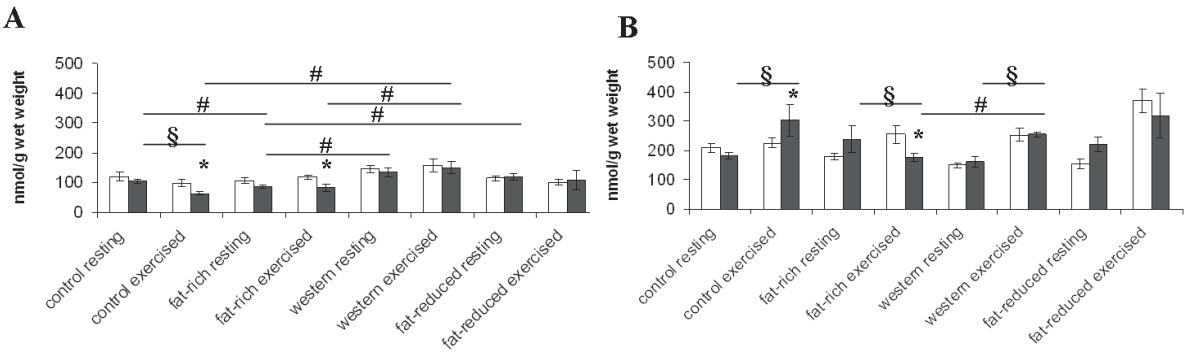


Figure 1: Free carnitine concentrations in skeletal muscle (A) and liver (B) of WT (n = 5) and VLCAD KO mice (n = 5). Free carnitine concentrations are presented in nmol/g wet weight. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± SEM. *p < 0.05 indicates significant differences between WT and VLCAD KO mice performed by Student´s t test, #p < 0.05 indicates significant differences between VLCAD KO mice in different dietary treated groups performed by two-way analysis of the variance (ANOVA) and §p < 0.05 indicating significant differences between rested and exercised VLCAD KO mice within one dietary treatment. Samples were analyzed in duplicate.

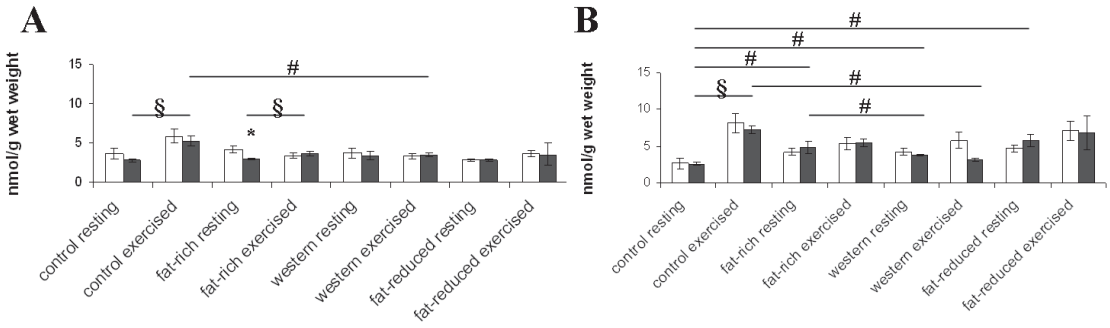


Figure 2: γ-butyrobetaine concentrations in skeletal muscle (A) and liver (B) of WT (n = 5) and VLCAD KO mice (n = 5). γ-butyrobetaine concentrations are presented in nmol/g wet weight. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± SEM. *p < 0.05 indicates significant differences between WT and VLCAD KO mice performed by Student´s t test, #p < 0.05 indicates significant differences between VLCAD KO mice in different dietary treated groups performed by two-way analysis of the variance (ANOVA) and §p < 0.05 indicating significant differences between rested and exercised VLCAD KO mice within one dietary treatment. Samples were analyzed in duplicate.

Interestingly, VLCAD KO mice fed with the fat-reduced diet had significantly higher acylcarnitine concentrations in skeletal muscle compared with the regular or the western type diet (Figure 3A). This was surprising, as the calorie content did not change compared with the

regular diet. However, the diet contained the highest amount of carbohydrates that are known as optimal energy source for short term activity, however not for longer-term exercise.

Our previous work demonstrated an increased lipogenesis during MCT-diet. An excessive supplementation of carbohydrates is known to have a similar effect (Boogaerts et al. 1984b). Therefore, liver lipid content was determined.

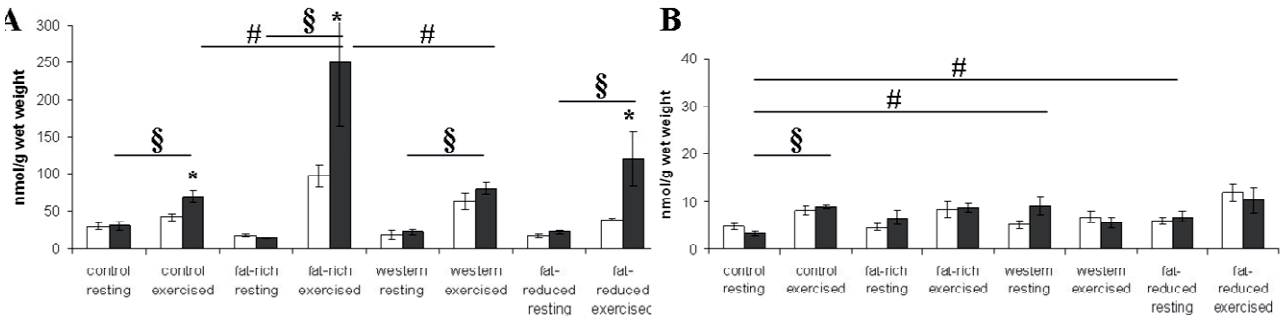


Figure 3: Acylcarnitine concentrations in skeletal muscle (A) and liver (B) of WT (n = 5) and VLCAD KO mice (n = 5). Acylcarnitine concentrations are presented in nmol/g wet weight. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values are means ± SEM. *p < 0.05 indicates significant differences between WT and VLCAD KO mice performed by Student´s t test, #p < 0.05 indicates significant differences between VLCAD KO mice in different dietary groups performed by two-way ANOVA and §p < 0.05 indicates significant differences between resting and exercised VLCAD KO mice within the same dietary group. Samples were analyzed in duplicate.

Hepatic acylcarnitines and lipid accumulation after exercise with dietary modification

During exercise, liver is less involved in energy production from mitochondrial β-oxidation, however responsible for sufficient carnitine supply through biosynthesis and the processing of lipids (Stein and Stein 1967; Vaz and Wanders 2002). Acylcarnitine concentrations generally remained very low in liver compared to acylcarnitine concentrations measured in skeletal muscle after exercise (Figure 3B). γ-butyrobetaine and free carnitine concentrations in VLCAD KO mice fed with the control or fat-rich diet significantly increased after exercise as free carnitine decreased during these conditions (Figure 1B, 2B).

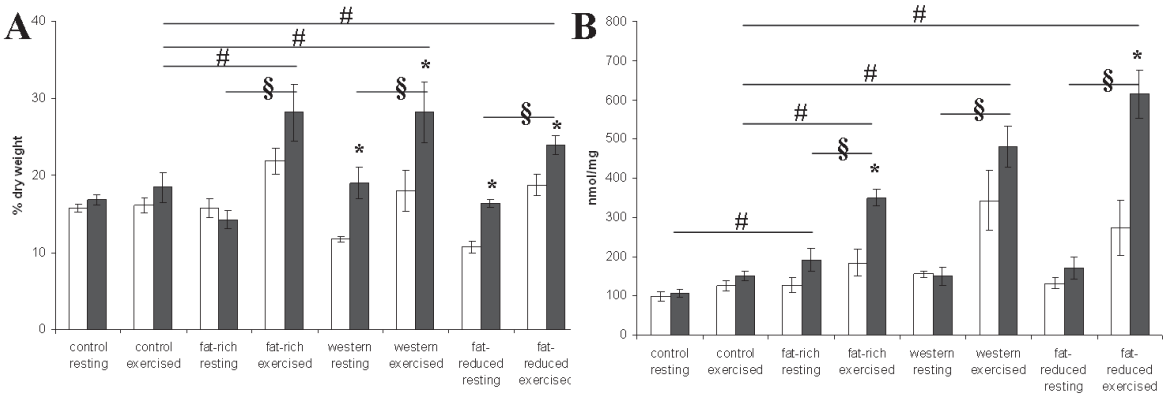


Figure 4: Intrahepatic lipid content (A) and triglyceride (TGA) content (B). Mean concentrations are expressed in dry weight (% dw) for the lipid content and nmol/mg for TGA content. The values are means ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice per dietary group under resting conditions and after exercise. *White bars* and *black bars* represent WT and VLCAD KO mice, respectively. Values were considered significant if $p < 0.05$. * indicates significant differences between WT and VLCAD KO mice within a group. # indicates significant differences between WT or VLCAD KO mice under different dietary conditions. § indicates significant differences between resting and exercised under the same dietary regimen. Significances were calculated by Student´s t test and two-way ANOVA with Bonferroni post-test.

Intrahepatic lipid content was significantly increased in VLCAD KO mice under all three dietary interventions after exercise in contrast to VLCAD KO mice fed with the control diet. A previous study showed an accumulation of intrahepatic lipid content up to 30 % of the dry weight in VLCAD KO mice under MCT-diet (Tucci et al. 2010). This finding was associated with fatty acid elongation and impaired lipid metabolism. In a similar manner, we detected an excessive liver TGA accumulation after exercise, which was particularly evident in VLCAD KO mice under the fat-reduced, carbohydrate-rich diet. This effect may be in part explained as physiological response to physical exercise. In fact, TGA and free fatty acids released by adipocytes during exercise are processed by the liver and again secreted into the blood to provide energy under situations of higher energy demand. It has been previously reported that liver fatty acid elongation is induced by high amounts of carbohydrates and by high fat feeding (Boogaerts et al. 1984a; Oosterveer et al. 2009). Whether fatty acid elongation and impaired lipid metabolism occur under the various fat modified diets and whether various glycogen content in the tissues plays a role needs to be further investigated.

In summary, the clinical phenotype of VLCAD KO mice was similar for all dietary interventions applied in this study. In fact, mice with the fat-reduced diet displayed highly elevated acylcarnitines and excessive liver TGA accumulation, which gave rise to the

assumption of increased lipogenesis. Fat-enriched diets, in contrast, did not worsen the clinical phenotype, however, also resulted in severe hepatic steatosis in mice.

Thus, a fat-reduced diet could not prevent myopathic symptoms and hepatic lipid accumulation in VLCAD KO mice after exercise. Sufficient energy supply appears to be essential to avoid myopathy as demonstrated for the western type diet, a high-caloric diet.

Acknowledgments

The study was financially supported by grants from the Deutsche Forschungsgemeinschaft (DFG, SP1125/1-1, SFB 575, and SFB 612) and from the Forschungskommission of the Medical Faculty of Heinrich-Heine-University Duesseldorf.

References

1. Arnold GL, Van Hove J, Freedenberg D, Strauss A, Longo N, Burton B, Garganta C, Ficicioglu C, Cederbaum S, Harding C, Boles RG, Matern D, Chakraborty P, Feigenbaum A (2009) A Delphi clinical practice protocol for the management of very long chain acyl-CoA dehydrogenase deficiency. Mol Genet Metab 96:85-90

2. Boogaerts JR, Malone-McNeal M, rchambault-Schexnayder J, Davis RA (1984a) Dietary carbohydrate induces lipogenesis and very-low-density lipoprotein synthesis. Am J Physiol 246:E77-E83

3. Boogaerts JR, Malone-McNeal M, rchambault-Schexnayder J, Davis RA (1984b) Dietary carbohydrate induces lipogenesis and very-low-density lipoprotein synthesis. Am J Physiol 246:E77-E83

4. Brown-Harrison MC, Nada MA, Sprecher H, Vianey-Saban C, Farquhar J, Jr., Gilladoga AC, Roe CR (1996) Very long chain acyl-CoA dehydrogenase deficiency: successful treatment of acute cardiomyopathy. Biochem Mol Med 58:59-65

5. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

6. FOLCH J, LEES M, SLOANE STANLEY GH (1957) A simple method for the isolation and purification of total lipides from animal tissues. J Biol Chem 226:497-509

7. Gillingham MB, Scott B, Elliott D, Harding CO (2006) Metabolic control during exercise with and without medium-chain triglycerides (MCT) in children with long-chain 3-hydroxy acyl-CoA dehydrogenase (LCHAD) or trifunctional protein (TFP) deficiency. Mol Genet Metab 89:58-63

8. Gregersen N, Bross P, Andresen BS (2004) Genetic defects in fatty acid beta-oxidation and acyl-CoA dehydrogenases. Molecular pathogenesis and genotype-phenotype relationships. Eur J Biochem 271:470-482

9. Liebig M, Schymik I, Mueller M, Wendel U, Mayatepek E, Ruiter J, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Neonatal screening for very long-chain acyl-coA dehydrogenase deficiency: enzymatic and molecular evaluation of neonates with elevated C14:1-carnitine levels. Pediatrics 118:1065-1069

10. Oosterveer MH, van Dijk TH, Tietge UJ, Boer T, Havinga R, Stellaard F, Groen AK, Kuipers F, Reijngoud DJ (2009) High fat feeding induces hepatic fatty acid elongation in mice. PLoS One 4:e6066

11. Papaioannou VE, Johnson RS (1993) Gene targeting: a practical approach. Oxford University Press, Oxford107-146

12. Primassin S, Ter VF, Mayatepek E, Spiekerkoetter U (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. Pediatr Res 63:632-637

13. Primassin S, Tucci S, Herebian D, Seibt A, Hoffmann L, Ter VF, Spiekerkoetter U (2010) Pre-exercise medium-chain triglyceride application prevents acylcarnitine accumulation in skeletal muscle from very-long-chain acyl-CoA-dehydrogenase-deficient mice. J Inherit Metab Dis 33:237-246

14. Roe CR, Sweetman L, Roe DS, David F, Brunengraber H (2002) Treatment of cardiomyopathy and rhabdomyolysis in long-chain fat oxidation disorders using an anaplerotic odd-chain triglyceride. J Clin Invest 110:259-269

15. Schulze-Bergkamen A, Okun JG, Spiekerkotter U, Lindner M, Haas D, Kohlmuller D, Mayatepek E, Schulze-Bergkamen H, Greenberg CR, Zschocke J, Hoffmann GF, Kolker S (2005) Quantitative acylcarnitine profiling in peripheral blood mononuclear cells using in vitro loading with palmitic and 2-oxoadipic acids: biochemical confirmation of fatty acid oxidation and organic acid disorders. Pediatr Res 58:873-880

16. Schymik I, Liebig M, Mueller M, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Pitfalls of neonatal screening for very-long-chain acyl-CoA dehydrogenase deficiency using tandem mass spectrometry. J Pediatr 149:128-130

17. Spiekerkoetter U (2007) Effects of a fat load and exercise on asymptomatic VLCAD deficiency. J Inherit Metab Dis 30:405

18. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de KH, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis

19. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U (2003) MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr 143:335-342

20. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

21. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van VN, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

22. Stein O, Stein Y (1967) Lipid synthesis, intracellular transport, storage, and secretion. I. Electron microscopic radioautographic study of liver after injection of tritiated palmitate or glycerol in fasted and ethanol-treated rats. J Cell Biol 33:319-339

23. Tucci S, Primassin S, ter Veld F, Spiekerkoetter U (2010) Medium-chain triglycerides impair lipid metabolism and induce hepatic steatosis in very long-chain acyl-CoA dehydrogenase deficient mice. Mol Genet Metab

24. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

25. Vaz FM, Wanders RJ (2002) Carnitine biosynthesis in mammals. Biochem J 361:417-429

26. Vockley J, Singh RH, Whiteman DA (2002) Diagnosis and management of defects of mitochondrial beta-oxidation. Curr Opin Clin Nutr Metab Care 5:601-609

Chapter 8

Corresponding increase in long-chain acyl-CoA and acylcarnitine after exercise in muscle from very long-chain acyl-CoA dehydrogenase-deficient mice

F. ter Veld, S. Primassin, L. Hoffmann, E. Mayatepek and U. Spiekerkoetter

J Lipid Res. 2009:50(8):1556-62

Abstract

Long-chain acylcarnitines accumulate in long-chain fatty acid oxidation defects, especially during periods of increased energy demand from fat. To test whether this increase in long-chain acylcarnitines in very long-chain acyl-CoA dehydrogenase knock-out mice ($VLCAD^{-/-}$) correlates with acyl-CoA content, we subjected wild-type (WT) and $VLCAD^{-/-}$ mice to forced treadmill running and analyzed muscle long-chain acyl-CoA and acylcarnitine with tandem mass spectrometry (MS/MS) in the same tissues.

After exercise, long-chain acyl-CoAs displayed a significant increase in muscle from $VLCAD^{-/-}$ mice (C16:0-CoA, C18:2-CoA and C18:1-CoA in sedentary $VLCAD^{-/-}$: 5.95 ± 0.33, 4.48 ± 0.51 and 7.70 ± 0.30 nmol · g^{-1} wet weight, respectively; in exercised $VLCAD^{-/-}$: 8.71 ± 0.42, 9.03 ± 0.93 and 14.82 ± 1.20 nmol · g^{-1} wet weight, respectively ($p < 0.05$). This increase in acyl-CoA in VLCAD-deficient muscle was paralleled by a significant increase in the corresponding chain length acylcarnitine. Furthermore, exercise resulted in significant lowering of the free carnitine pool in $VLCAD^{-/-}$ muscle.

In conclusion, this is the first study demonstrating that acylcarnitines and acyl-CoA directly correlate and concomitantly increase after exercise in VLCAD-deficient muscle.

Introduction

Very-long-chain acyl-CoA dehydrogenase (VLCAD or ACADVL, EC 1.3.99.3) catalyzes the first step in the β-oxidation pathway of long (C_{14} or longer) chain fatty acids. Deficiency of this enzyme severely impairs the oxidation of long-chain fatty acids (Wanders et al. 1999). Mutations in the VLCAD gene lead to clinical symptoms in children and young adults. Physiological stressors such as fasting, exercise, and viral illnesses are triggers of disease crises (Mathur et al. 1999; Strauss et al. 1995). NBS programs (Liebig et al. 2006b; Schymik et al. 2006) have, however, drastically improved long-term survival and a great number of patients have remained asymptomatic with proper preventive measures during the first years of life (Liebig et al. 2006b). However, as there is no prevalent VLCAD mutation and VLCAD mutations can not be correlated to residual VLCAD activity, as measured in fibroblasts or lymphocytes, the late-onset disease phenotype varies greatly (Vianey-Saban et al. 1998). NBS programs are thus identifying more and more patients carrying VLCAD mutations whose clinical outcome can not be predicted at the time of diagnosis. Therefore, the effect of

possible disease triggers such as exercise-stress and their resulting metabolic phenotype, i.e. acylcarnitine and/or acyl-CoA accumulation, need to be identified.

With the generation of a transgenic VLCAD-deficient (KO) mouse, that displays a stress-induced phenotype similar to that of humans (Exil et al. 2003; Spiekerkoetter et al. 2004), new possibilities arise to study the effect of physiological stressors, such as fasting and exercise, at tissue-level. In these mice it was shown that they have impaired Ca^{2+} handling (Werdich et al. 2007) and heart rate dysfunction (Exil et al. 2006). VLCAD deficient soleus muscle showed an increase in mitochondrial density and mitochondria were more heterogeneous in size and appeared disorganized (Exil et al. 2003). Similar to VLCAD deficient patients, the metabolic VLCAD KO mouse phenotype displays an accumulation of fat droplets (Exil et al. 2003) and long-chain acylcarnitines (Liebig et al. 2006a) in muscle.

Acylcarnitines are known to have unspecific cytotoxic effects (Primassin et al. 2008; Bonnet et al. 1999) and accumulate in VLCAD-deficient patients during crisis due to insufficient oxidation capacity of long-chain fatty acids (Spiekerkoetter et al. 2003). As cytosolic carnitine palmitoyltransferase (CPT) I and mitochondrial CPT-II catalyze the forward and reverse conjugation of acyl-CoA to carnitine, increased long-chain acylcarnitine content in VLCAD KO tissue is expected, but not proven, to be accompanied by increased long-chain acyl-CoA levels. Long-chain acyl-CoA accumulation in tissue may well result in lower mitochondrial respiration via inhibition the mitochondrial adenine nucleotide translocator in the µM range (Ciapaite et al. 2006).

Taken together, although the accumulation of long-chain acylcarnitines in VLCAD deficient patients and in the corresponding mouse model has been demonstrated, an increase in long-chain acyl-CoA levels has, so far, only been *assumed*.

For the first time, we are now able to simultaneously measure acyl-CoA and acylcarnitines in muscle from VLCAD-deficient mice after exercise stress. Both measurements are performed by ESI-MS/MS in the same muscle sample and, therefore, allow accurate and direct correlation of both metabolites in sedentary and exercised muscle.

Materials and Methods

Reagents

Heptadecanoyl-CoA (C17-CoA) was purchased from Sigma (Deisenhofen, Germany) as Li^+ salts and stored at -20 °C. Internal standard heptadecanoyl-CoA was prepared by dissolving in methanol to obtain a concentration of 2.5 mM and stored at -80°C until use.

Animals

VLCAD-deficient mice were generated as described in detail previously (Exil et al. 2003). Experiments were performed on second- to third-generation intercrosses of C57BL6+129sv VLCAD genotypes. Littermates served as controls and genotyping of mice was performed as described previously (Spiekerkoetter et al. 2005). Mice were fed with a standard chow diet (MZ Extrudat from sniff® containing 4.5 % w/w crude fat, corresponding to 13% of total metabolizable energy according to the Atwater System) and received tap water ad libitum. As mice are nocturnal animals, treadmill running was performed during the dark cycle. All animal studies were in accordance with the Heinrich-Heine-University Medical Center and Institutional Animal Care and Use Committee guidelines.

Training protocol

Four-month-old WT and VLCAD KO animals ran 120 min on a Columbus Instruments Simplex II metabolic rodent treadmill, consisting of four individual lanes, without inclination, with shock plate incentive (3 Hz, 200 msec, 160 V, 1.5 mA). Mice were placed in an exercise chamber, and after an adaptation period of 15 min, initial belt speed was set to 4 m/min and increased every 5 min by 2 m/min to a maximum of 15 m/min. Mice ran until they displayed signs of exhaustion (>2 s spent on the shocker plate without attempting to re-engage the treadmill). To test for long-term alterations in skeletal muscle mitochondrial content, WT and VLCAD KO animals ran for 5 days at an average belt-speed of 8 m/min. The sedentary group consisted of 4 wild-type and 5 VLCAD KO animals and the exercised group consisted of 4 wild-type and 4 VLCAD KO animals.

Acyl-CoA analysis

Hind leg skeletal muscle (100 – 200 mg) was taken up in five volumes of extraction buffer (1:1:1 iso-propanol / acetonitrile / 10 mM NH_4Ac, pH=5.0) and minced on ice with scissors. Five nmoles of heptadecanoyl-CoA (C17:0-CoA) were added as internal standard and the tissue was homogenized using an Ultra-Turrax®. Next, the homogenate was centrifuged at 1000 x *g* for 5 min., the supernatant was collected and the pellet was re-homogenized in five volumes of extraction buffer. The homogenate was centrifuged at 1000 x

g for 5 min. and the supernatants were pooled. Next, the pooled supernatants were centrifuged at 1000 x *g* for 5 min. to remove any remaining cellular debris. For analysis, sample volumes were reduced by approx. 50% using evaporation at 40 °C under air.

For LC-tandem MS analysis, a Waters 2795 Alliance HPLC system (Waters, Milford, UK), equipped with a thermostated autosampler, was used for solvent delivery and sample introduction. Assay samples were placed in a cooled sample tray and 20 µL were injected onto a reversed-phase Symmetry C18 column (3.5µ; 7.5 x 4.6 mm; WAT066224; Waters), protected by a guard column (SecurityGuard C18 ODS; 4 x 2.0 mm; Phenomenex). C18:2-CoA, C18:1-CoA, C16:0-CoA and C17:0-CoA were eluted isocratically with 55 % (v/v) acetonitrile in 10 mM NH_4Ac (pH=6.5) at a flow rate of 0.6 mL/min. The eluate was delivered into a Quattro Micro API tandem mass spectrometer (Micromass, Cambridge, UK) with an ESI probe in positive-ion mode. The waste-valve was used to discard early-eluting salts from contaminating the mass spectrometer. The injection interval was 5 min. Nitrogen was used as drying gas at a flow rate of 700 L/h. The collision energy using argon as collision gas was 35 eV. The declustering potential was 45 V, the ion source temperature was 120°C. Compounds were detected in the multiple-reaction monitoring (MRM) mode. A representative chromatogram with (A) and without (B) internal standard C17:0-CoA is shown in Figure 1. Specific transitions were used for each metabolite, but HPLC separation remained necessary to remove interfering compounds, such as salts, present in muscle extracts, thereby preventing tandem MS apparatus contamination.

Acylcarnitine and carnitine analysis

In tissues, analysis of carnitine and acylcarnitines was performed according to van Vlies et al. (van Vlies et al. 2005). Briefly, 50 mg of blotted skeletal muscle was lyophilized for 12 h, including internal standards (16.25 nmoles $[^2H_3]$carnitine and 0.05 nmoles $[^2H_3]C_{16}$-acylcarnitine). The lyophilized tissues were pulverized and dissolved in 1 mL of 80% v/v acetonitrile. After homogenization and centrifugation the supernatant was dried. Finally, carnitine and acylcarnitines were analyzed by ESI-MS/MS as their butyl esters and resuspended in 100 µL ACN/H_2O (50/50% v/v).

Citrate synthase activity

Citrate synthase enzyme activity of tissue homogenates was determined to characterize Krebs cycle activity as described previously (Shepherd and Garland 1969). Samples were assayed in duplicate in flat-bottom microtiter plates, the absorption coefficient was adjusted to the reaction volume (i.e. calculated light path). The media used in the above assays were adjusted to 0.1% Triton X-100 to obtain maximal enzyme activities in tissue homogenates.

Figure 1

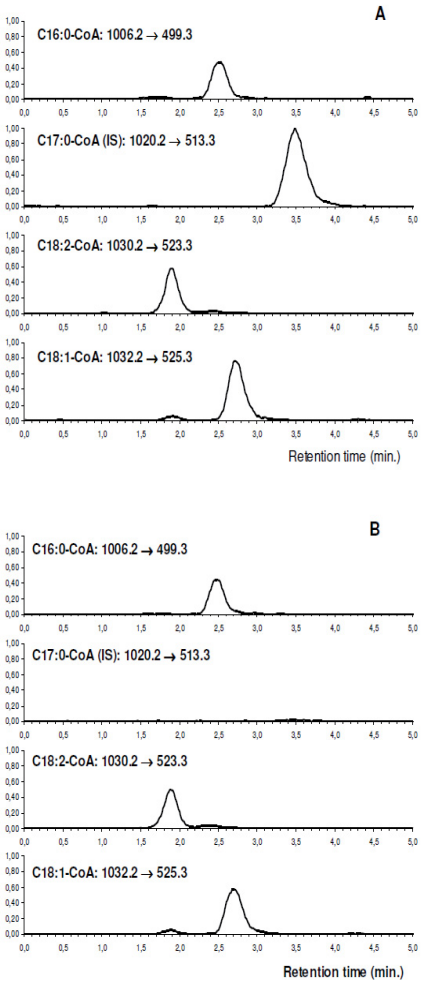


Figure 1: Multiple Reactant Monitoring (MRM) chromatograms of a mouse muscle extracts containing long-chain fatty acyl-CoA esters with (A) and without (B) internal standard C17:0-CoA. Monitored transitions, in order of elution: linoleoyl-coenzyme A (C18:2-CoA): 1030.2 → 523.3; palmitoyl-coenzyme A (C16:0-CoA): 1006.2 → 499.3; oleoyl-coenzyme A (C18:1-CoA): 1032.2 → 526.3; heptadecanoyl-coenzyme A (C17:0-CoA) 1020.2 → 513.3.

Cytochrome c oxidase subunit 3 mRNA expression

Expression of mitochondrial DNA encoded cytochrome c oxidase subunit 3 (cox3) mRNA expression was performed as described previously (Schafer et al. 2007) to determine the relative mitochondrial quantity. β-Actin mRNA expression was unaffected by our experimental design and was used as a reference. Cox4 mRNA/cDNA concentration was normalized for the concentrations of β-actine mRNA/cDNA in the same sample. Values are stated as percentage of the sedentary control samples.

Protein determination

Protein concentration of tissue homogenates was determined by the BCA assay (Pierce). The BCA reagent was supplemented with 0.1% (v/v) Triton X-100. BSA was used as standard.

Data analysis and statistics

Data were acquired and analysed using MassLynx NT v4.0 software (Micromass, UK). Data were analyzed with Origin 6.0 (Microcal Software Inc., Northhampton, MA, U.S.A.). If not stated otherwise, reported data are presented as arithmetic means ± standard deviation (SD) with *n* denoting the number of animals. Statistical analyses were performed using Student's *t* test. Differences between means were considered significant if $p < 0.05$.

Results

Animal condition and mitochondrial content

In contrast to WT mice, VLCAD KO mice had difficulties to maintain a running speed of 15 m/min for the full 120 min, resulting in shorter running times for VLCAD KO mice (Table 1). To stress the mice to their maximal capacity, different running times were needed. Body weights were similar for sedentary WT and VLCAD KO mice (Table 1). Possible proliferation of muscle mitochondria, due to VLCAD-deficiency and/or exercise, was studied by measuring indirect marker enzyme citrate synthase and the expression of cytochrome c oxidase subunit 3 (cox3) mRNA. Muscle citrate synthase activity (in mU · mg protein^{-1}) and cox3 expression were not significantly different in WT as well as VLCAD KO mouse skeletal muscle (Table 1).

Table 1

Animal condition, exercise capacity and muscle mitochondrial content, expressed as citrate synthase activity (mU · mg protein^{-1}) and Cox3 mRNA expression (as % of wild-type).

	sedentary		exercised	
	wild-type	VLCAD$^{-/-}$	wild-type	VLCAD$^{-/-}$
	(n = 4) (n = 5)	(n = 4)	(n = 4)	
Body weight (g)	25.5 ± 4.2	30.0 ± 8.1	30.7 ± 9.8	24.56 ± 3.2
Running time (min)	-	-	120	95
Citrate synthase activity	244.7 ± 28.0	242.3 ± 59.1	258.6 ± 14.0	271.7 ± 15.4
Cox3 mRNA (%)	100	99.0 ± 2.7	97.5 ± 2.9	99.5 ± 1.9

Muscle acyl-CoA and acylcarnitine content

We first measured muscle acylcarnitine and acyl-CoA content in WT and VLCAD KO mice under sedentary conditions. Overall levels of long-chain acylcarnitines and acyl-CoA were similar in sedentary WT and VLCAD KO muscle, with the exception of muscle C18:1-carnitine content, which was significantly higher in VLCAD KO mice, compared to WT mice (11.29 ± 3.26 (n = 5) and 7.01 ± 1.74 (n = 4) nmol · g^{-1} wet weight, respectively; $p < 0.05$) (Figure 2B).

After exercise stress, consisting of treadmill running at 15 m/min, analysis of long-chain acyl-CoAs revealed a significant increase in VLCAD KO muscle (C16:0-CoA, C18:2-CoA and C18:1-CoA in sedentary VLCAD KO mice: 5.95 ± 0.74, 4.48 ± 1.14 and 7.70 ± 0.67 nmol · g^{-1} wet weight, respectively (n = 5); in exercised VLCAD KO mice: 8.71 ± 0.84, 9.03 ± 1.86 and 14.82 ± 2.40 nmol · g^{-1} wet weight, respectively; $p < 0.05$; n = 4) (Figure 2A). This exercise-induced increase in VLCAD-deficient muscle was paralleled by a similar and significant increase in corresponding acylcarnitines with the same acyl-chain length (C16:0-carnitine, C18:2-carnitine and C18:1-carnitine in sedentary VLCAD KO mice: 8.26 ± 3.29, 6.22 ± 2.78 and 11.29 ± 3.27 nmol · g^{-1} wet weight, respectively (n = 5); in exercised VLCAD KO mice: 17.91 ± 4.62, 14.58 ± 3.08 and 26.50 ± 9.16 nmol · g^{-1} wet weight, respectively; $p < 0.05$; n = 4) (Figure 2B).

Figure 2

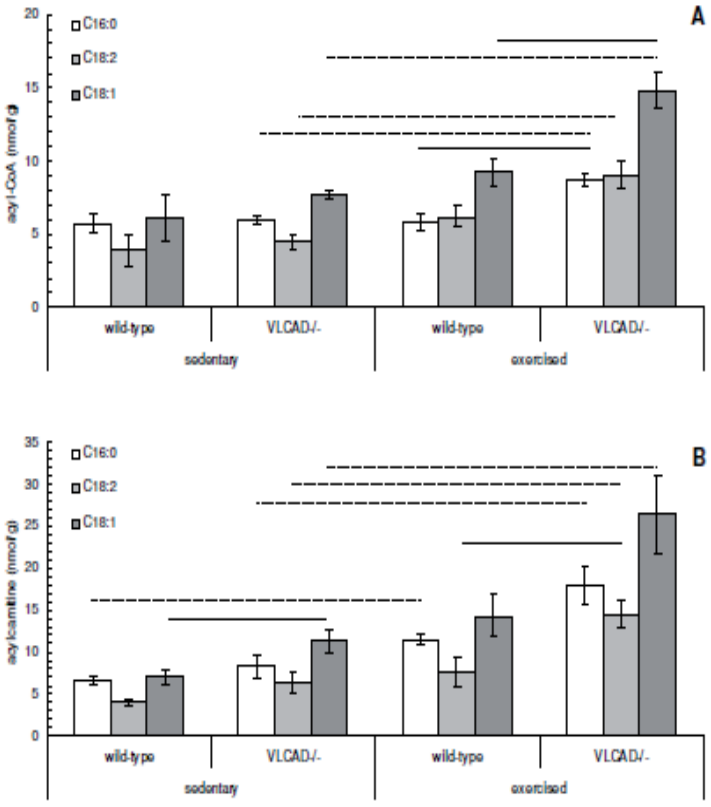


Figure 2: Muscle acyl-CoA (A) and acylcarnitine (B) levels in sedentary WT (n = 4) and VLCAD$^{-/-}$ (n = 5) and exercised WT (n = 4) and VLCAD$^{-/-}$ (n = 4) mice. Values are means ± SEM. (—), $p < 0.05$ compared to WT. (···), $p < 0.05$ compared to sedentary mice.

Wild-type mice showed a significant increase in C16:0-carnitine after 2 hours of belt running, compared to sedentary palmitoyl-carnitine muscle content (6.64 ± 1.14 and 11.46 ± 1.08 nmol · g^{-1} wet weight for sedentary vs. exercised WT mice; $p < 0.05$; n = 4; Figure 2B). We observed no significant differences between groups in muscle medium-chain octanoyl (C8:0)-carnitine content (data not shown).

Muscle free L-carnitine content

Exercise resulted in lowering of carnitine in both WT and VLCAD KO muscle, this reduction was significant in VLCAD KO mice, but not in WT mice (carnitine muscle levels in sedentary and exercised VLCAD KO mice: 154.3 ± 35.8 (n = 5) and 106.5 ± 13.6 (n = 4) nmol · g^{-1} wet weight ($p < 0.05$), respectively, vs. sedentary and exercised WT: 199.3 ± 40.4 (n = 4) and 134.7 ± 37.0 (n = 4) nmol · g^{-1} wet weight, respectively).

Discussion

In the present study, long-chain acylcarnitines and acyl-CoAs were measured to monitor the effect of exercise stress on VLCAD-deficient muscle. We were able to show for the first time that exercise stress, consisting of treadmill running, resulted in a significant, corresponding increase in C16:0, C18:1 and C18:2-carnitine and -CoA in VLCAD KO mouse muscle. Accumulation of long-chain acylcarnitines is well documented and has been implicated in the development of rhabdomyolysis in VLCAD-deficient patients (Smelt et al. 1998). However, it is unknown whether, and to what extent, this increase in long-chain acylcarnitines is mirrored by an increase in acyl-CoA esters of corresponding chain-length. The mouse model of VLCAD-deficiency also displays acylcarnitine accumulation in tissues during fasting and exercise stress (Spiekerkoetter et al. 2005) and is therefore a suitable animal model to study the effects of VLCAD-deficiency at a cellular level.

First, we measured if mitochondrial content was altered in exercised VLCAD-deficient muscle. This is of importance because in heart muscle approx. 95% of the cellular CoA pool is located inside mitochondria (Idell-Wenger et al. 1978). Previous studies have shown that mitochondrial density increases in *oxidative* soleus and heart muscle of VLCAD-deficient mice (Exil et al. 2003). In *glycolytic* skeletal gastrocnemius and extensor digitorum longus muscle from WT and VLCAD KO mice, we compared mitochondrial content, based on citrate synthase enzyme activity, as indirect marker, and Cox3 mRNA expression, and observed that, in contrast to oxidative muscle, mitochondrial content was identical for WT and VLCAD KO

muscle at rest and did not increase due to exercise (Table 1). The observation of similar citrate synthase activity in sedentary and exercised murine skeletal muscle is in line with previous work from (Jeneson et al. 2007), where treadmill running had no effect on glycolytic fast-twitch *extensor digitorum longus* citrate synthase content. Taken together, these data suggest that in oxidative muscle mitochondrial proliferation occurs as compensatory mechanism, on the energy supply side of cellular energy homeostasis, in an effort to counteract impaired fatty acid oxidation. In contrast, in glycolytic skeletal muscles this cellular remodelling appears not to take place.

Long-chain acylcarnitine content, by measuring C16:0, C18:1 and C18:2-carnitine, was slightly higher in VLCAD KO striated skeletal muscle under sedentary conditions, as published previously (Spiekerkoetter et al. 2005). However, this increase was only significant for C18:1-carnitine, an observation also reported by Cox et al. (Cox et al. 2001). In sedentary VLCAD KO muscle, long-chain C16:0, C18:1 and C18:2 acyl-CoAs were not increased, compared to WT muscle. The absolute content of long-chain acyl-CoA (in nmol $\cdot$ g^{-1} wet weight) in WT mouse muscle was comparable to reported values in literature, obtained with both HPLC and mass spectrometry techniques (Ciapaite et al. 2007; Kalderon et al. 2002; Sun et al. 2006; Woldegiorgis et al. 1985; Yu et al. 2002).

Exercise stress significantly increased long-chain acyl metabolites, with C18:1 chain-length having the most pronounced effect. Elevation of C18:1-carnitine and C18:1-CoA was approximately 4-fold and 3-fold, respectively, in exercised VLCAD KO muscle, as compared to sedentary WT muscle. Unlike WT exercised muscle all long-chain acyl CoA esters significantly increased in VLCAD KO muscle upon exercise (Figure 2), illustrating the impact of workload on the VLCAD-deficient phenotype and the extent of activated fatty acid oxidation. In addition, it appears that during periods of elevated workload, the carnitine pool of VLCAD-deficient muscle can no longer adequately scavenge acyl moieties, resulting in a significant increase in muscle long-chain acyl-CoA esters. Importantly, this exercise-induced fast increase in long-chain acyl-CoA esters is absent in WT muscle.

The effect of enhanced workload on long-chain acyl-CoA ester levels can be better illustrated by plotting the exercise-induced increase in acyl-CoA and acylcarnitine as percentage of sedentary levels (Figure 3). We clearly observe that exercise results in a stronger accumulation of acylcarnitines than acyl-CoAs in both WT as well as VLCAD KO mice. And although the magnitude by which both long-chain acylcarnitines and acyl-CoAs increase is higher in VLCAD KO as compared to WT muscle (Figure 3), importantly, in relation to each other these acyl CoA esters appear to be well equilibrated by the action of

carnitine acyltransferases. This is observed in WT *and* VLCAD KO muscle, as the exercise-induced increase in long-chain acylcarnitines is approx. 2.5-fold higher than the increase in corresponding acyl-CoA esters (Figure 3). The proposed role of the carnitine pool functioning as acyl scavenger is, furthermore, supported by our measurements of muscle free carnitine, demonstrating a significant drop in free carnitine in VLCAD KO muscle upon exercise. Thus, during periods of elevated workload, the acylation state of the cytosolic carnitine pool increases more than that of the mitochondrial pool, indicative of acyl-compounds being exported out of the muscle cell (Ramsay and Arduini 1993). This observation of course fits well with the increased levels of long-chain acylcarnitines in serum and blood from VLCAD KO mice (Spiekerkoetter et al. 2005; Cox et al. 2001), as acylcarnitines in blood would accumulate over time due to cellular efflux.

Figure 3

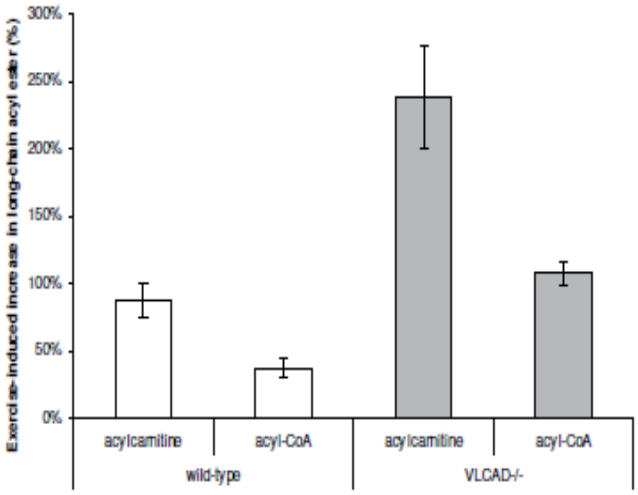


Figure 3: Exercise-induced increase, as percentage of sedentary levels, in long-chain (C16:0, C18:2 and C18:1) CoA and carnitine esters in WT (n = 4) and VLCAD$^{-/-}$ (n = 5) mouse muscle. Values are means ± SEM. Significances not tested.

Acylcarnitines are known to have unspecific cytotoxic effects (Primassin et al. 2008) and long-chain acyl-CoAs are known to inhibit the mitochondrial adenine nucleotide translocator (Ciapaite et al. 2006), however, further studies have to determine which of these two metabolites is more cytotoxic *in-vivo*. Thus, in addition to impaired energy production from long-chain fatty acids due to the enzyme defect itself, toxic effects of accumulating acylcarnitines and acyl-CoA esters may play an important role in disease pathogenesis. Interestingly, detailed histological investigation of VLCAD KO mouse heart has indeed demonstrated an increase in degenerative fibers, collagen deposition and vacuolated myocytes, which may be indicative of cytotoxicity (Exil et al. 2003). These cellular

alterations in VLCAD-deficient heart were accompanied by changes in mitochondrial ultrastructure, an adaptive response also observed in VLCAD KO *soleus* muscle cells (Exil et al. 2003). However, it is currently still unknown whether these changes are the direct consequence of accumulating (long-chain) acylcarnitines and acyl-CoA esters. Of note, calculation of absolute cytosolic concentrations based on tissue content (in nmol $\cdot$ g^{-1}) should be treated with caution because free long-chain fatty acid and acyl-CoA concentrations are buffered by fatty acid and acyl-CoA binding proteins inside the cell (Faergeman and Knudsen 1997), thereby lowering the concentration on free acyl compounds.

In conclusion, we demonstrate for the first time that long-chain acylcarnitines and acyl-CoA esters increase and directly correlate in VLCAD KO mouse muscle after exercise. However, the question whether exercise-induced accumulation of acyl esters is an important factor in disease pathogenesis can not be answered at this time.

Acknowledgements

We thank Dr. M.D. Laryea, Department of General Pediatrics, Heinrich-Heine-University, Duesseldorf, Germany for expert advice on liquid-chromatography analysis. We are grateful to Mrs. G. Schmitz and Mr. A. Vogt for technical assistance. The study was financially supported by grants from the Forschungskommission of the Medical Faculty of the Heinrich-Heine-University Düsseldorf, the Deutsche Forschungsgemeinschaft (DFG, SP1125/1-1) and from the Heinrich-Heine-University Düsseldorf (SFB 575).

References

1. Bonnet D, Martin D, Pascale DL, Villain E, Jouvet P, Rabier D, Brivet M, Saudubray JM (1999) Arrhythmias and conduction defects as presenting symptoms of fatty acid oxidation disorders in children. Circulation 100:2248-2253

2. Ciapaite J, Bakker SJ, Diamant M, van Eikenhorst G, Heine RJ, Westerhoff HV, Krab K (2006) Metabolic control of mitochondrial properties by adenine nucleotide translocator determines palmitoyl-CoA effects. Implications for a mechanism linking obesity and type 2 diabetes. FEBS J 273:5288-5302

3. Ciapaite J, Bakker SJ, van EG, Wagner MJ, Teerlink T, Schalkwijk CG, Fodor M, Ouwens DM, Diamant M, Heine RJ, Westerhoff HV, Krab K (2007) Functioning of

oxidative phosphorylation in liver mitochondria of high-fat diet fed rats. Biochim Biophys Acta 1772:307-316

4. Cox KB, Hamm DA, Millington DS, Matern D, Vockley J, Rinaldo P, Pinkert CA, Rhead WJ, Lindsey JR, Wood PA (2001) Gestational, pathologic and biochemical differences between very long-chain acyl-CoA dehydrogenase deficiency and long-chain acyl-CoA dehydrogenase deficiency in the mouse. Hum Mol Genet 10:2069-2077

5. Exil VJ, Gardner CD, Rottman JN, Sims H, Bartelds B, Khuchua Z, Sindhal R, Ni G, Strauss AW (2006) Abnormal mitochondrial bioenergetics and heart rate dysfunction in mice lacking very-long-chain acyl-CoA dehydrogenase. Am J Physiol Heart Circ Physiol 290:H1289-H1297

6. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

7. Faergeman NJ, Knudsen J (1997) Role of long-chain fatty acyl-CoA esters in the regulation of metabolism and in cell signalling. Biochem J 323 (Pt 1):1-12

8. Idell-Wenger JA, Grotyohann LW, Neely JR (1978) Coenzyme A and carnitine distribution in normal and ischemic hearts. J Biol Chem 253:4310-4318

9. Jeneson JA, de Snoo MW, Verlinden NA, Joosten BJ, Doornenbal A, Schot A, Everts ME (2007) Treadmill but not wheel running improves fatigue resistance of isolated extensor digitorum longus muscle in mice. Acta Physiol (Oxf) 190:151-161

10. Kalderon B, Sheena V, Shachrur S, Hertz R, Bar-Tana J (2002) Modulation by nutrients and drugs of liver acyl-CoAs analyzed by mass spectrometry. J Lipid Res 43:1125-1132

11. Liebig M, Gyenes M, Brauers G, Ruiter JP, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006a) Carnitine supplementation induces long-chain acylcarnitine production--studies in the VLCAD-deficient mouse. J Inherit Metab Dis 29:343-344

12. Liebig M, Schymik I, Mueller M, Wendel U, Mayatepek E, Ruiter J, Strauss AW, Wanders RJ, Spiekerkoetter U (2006b) Neonatal screening for very long-chain acyl-coA dehydrogenase deficiency: enzymatic and molecular evaluation of neonates with elevated C14:1-carnitine levels. Pediatrics 118:1065-1069

13. Mathur A, Sims HF, Gopalakrishnan D, Gibson B, Rinaldo P, Vockley J, Hug G, Strauss AW (1999) Molecular heterogeneity in very-long-chain acyl-CoA dehydrogenase deficiency causing pediatric cardiomyopathy and sudden death. Circulation 99:1337-1343

14. Primassin S, ter Veld F, Mayatepek E, Spiekerkoetter U (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. Pediatr Res

15. Ramsay RR, Arduini A (1993) The carnitine acyltransferases and their role in modulating acyl-CoA pools. Arch Biochem Biophys 302:307-314

16. Schafer C, Hoffmann L, Heldt K, Lornejad-Schafer MR, Brauers G, Gehrmann T, Garrow TA, Haussinger D, Mayatepek E, Schwahn BC, Schliess F (2007) Osmotic regulation of betaine homocysteine-S-methyltransferase expression in H4IIE rat hepatoma cells. Am J Physiol Gastrointest Liver Physiol 292:G1089-G1098

17. Schymik I, Liebig M, Mueller M, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Pitfalls of neonatal screening for very-long-chain acyl-CoA dehydrogenase deficiency using tandem mass spectrometry. J Pediatr 149:128-130

18. Shepherd D, Garland PB (1969) The kinetic properties of citrate synthase from rat liver mitochondria. Biochem J 114:597-610

19. Smelt AH, Poorthuis BJ, Onkenhout W, Scholte HR, Andresen BS, van Duinen SG, Gregersen N, Wintzen AR (1998) Very long chain acyl-coenzyme A dehydrogenase deficiency with adult onset. Ann Neurol 43:540-544

20. Spiekerkoetter U, Sun B, Zytkovicz T, Wanders R, Strauss AW, Wendel U (2003) MS/MS-based newborn and family screening detects asymptomatic patients with very-long-chain acyl-CoA dehydrogenase deficiency. J Pediatr 143:335-342

21. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

22. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van Vlies N, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

23. Strauss AW, Powell CK, Hale DE, Anderson MM, Ahuja A, Brackett JC, Sims HF (1995) Molecular basis of human mitochondrial very-long-chain acyl-CoA dehydrogenase deficiency causing cardiomyopathy and sudden death in childhood. Proc Natl Acad Sci U S A 92:10496-10500

24. Sun D, Cree MG, Wolfe RR (2006) Quantification of the concentration and 13C tracer enrichment of long-chain fatty acyl-coenzyme A in muscle by liquid chromatography/mass spectrometry. Anal Biochem 349:87-95

25. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

26. Vianey-Saban C, Divry P, Brivet M, Nada M, Zabot MT, Mathieu M, Roe C (1998) Mitochondrial very-long-chain acyl-coenzyme A dehydrogenase deficiency: clinical characteristics and diagnostic considerations in 30 patients. Clin Chim Acta 269:43-62

27. Wanders RJ, Vreken P, den Boer ME, Wijburg FA, van Gennip AH, Ijlst L (1999) Disorders of mitochondrial fatty acyl-CoA beta-oxidation. J Inherit Metab Dis 22:442-487

28. Werdich AA, Baudenbacher F, Dzhura I, Jeyakumar LH, Kannankeril PJ, Fleischer S, LeGrone A, Milatovic D, Aschner M, Strauss AW, Anderson ME, Exil VJ (2007)

Polymorphic ventricular tachycardia and abnormal Ca2+ handling in very-long-chain acyl-CoA dehydrogenase null mice. Am J Physiol Heart Circ Physiol 292:H2202-H2211

29. Woldegiorgis G, Spennetta T, Corkey BE, Williamson JR, Shrago E (1985) Extraction of tissue long-chain acyl-CoA esters and measurement by reverse-phase high-performance liquid chromatography. Anal Biochem 150:8-12

30. Yu C, Chen Y, Cline GW, Zhang D, Zong H, Wang Y, Bergeron R, Kim JK, Cushman SW, Cooney GJ, Atcheson B, White MF, Kraegen EW, Shulman GI (2002) Mechanism by which fatty acids inhibit insulin activation of insulin receptor substrate-1 (IRS-1)-associated phosphatidylinositol 3-kinase activity in muscle. J Biol Chem 277:50230-50236

Chapter 9

Discussion

VLCAD-deficiency is an inherited enzyme deficiency concerning mitochondrial β-oxidation. As fatty acids are an important source of energy in humans and most animals, insufficient fat metabolism may have severe clinical consequences. There have been many approaches to assist patients suffering from VLCAD-deficiency with preventive and therapeutic measures to avoid critical and even life-threatening situations (Kerner and Hoppel 1998; Vockley et al. 2002). There is increasing evidence that different pathomechanisms are responsible for the heterogeneous clinical phenotypes of VLCAD deficiency. In this thesis, different aspects of pathophysiology were studied, one of them regarding carnitine homeostasis.

In order to determine carnitine metabolites in blood we used an ESI-MS/MS method, similar to that performed in NBS. We modified this method for the analysis of carnitine / acylcarnitine metabolites in tissues of the VLCAD KO mouse model. This animal model has formerly been proven to be adequate to investigate the metabolic status in tissues because of similar phenotypes under catabolic stress compared with human patients (Exil et al. 2003; Spiekerkoetter et al. 2004; Spiekerkoetter et al. 2005; Spiekerkoetter et al. 2006).

Carnitine supplementation and biosynthesis

In the past, carnitine supplementation was considered for therapy in the treatment of VLCAD-deficiency. In fact, carnitine is a very important component in the transport of long-chain fatty acids through the mitochondrial membranes and secondary deficiency occurs in fatty acid oxidation defects due to loss of free carnitine in parallel with accumulation of long-chain acylcarnitines in these disorders (Kerner and Hoppel 1998; Ramsay et al. 2001; Winter 2003). In chapter 2 we demonstrated the effect of L-carnitine supplementation on exercise-induced changes in free carnitine and acylcarnitine concentrations in skeletal muscle, liver and blood from WT and VLCAD KO mice. Previous experiments in VLCAD KO mice showed that the dosage of 100 $mg*kg^{-1}*day^{-1}$ L-carnitine used in humans (Liebig et al. 2006) did not prevent low free carnitine levels after exercise. Therefore, the mice were supplemented with double the dosage of L-carnitine compared with usual clinical use. In order to gain more insight into the intrinsic pathway of carnitine biosynthesis, we also analyzed γ-butyrobetaine in murine skeletal muscle and liver, in addition to the metabolic steps involved in the consumption of carnitine. As a very important observation, we could demonstrate that in the VLCAD KO mouse model long-term supplementation of L-carnitine induces acylcarnitine production, without replenishing low tissue carnitine concentrations. As acylcarnitines are considered potentially toxic, this observation has significant consequences

for treatment recommendations. In addition, we could also show that endogenous carnitine biosynthesis is able to replenish low free carnitine tissue levels in non-supplemented mice after 24 h of regeneration (Chapter 2; Primassin et al. 2008).

With respect to patients, it still remains unclear whether carnitine supplementation helps to prevent secondary carnitine deficiency, especially with respect to tissue levels (Primassin et al. 2008). Regular measurements in tissues of patients are not possible and the usefulness of carnitine blood levels for monitoring during treatment remains questionable. In chapter 3 we evaluated dried blood spots (DBS) and plasma samples from numerous patients to directly compare these two blood compartments with respect to the carnitine status. It was of great interest whether it is also possible in blood to determine whether endogenous carnitine biosynthesis is activated and contributes to the carnitine concentration in blood. We studied whether γ-butyrobetaine, the precursor of carnitine, can also be used as a marker for carnitine biosynthesis and the extent of carnitine deficiency in blood. In the current study, we quantified concentrations of free carnitine and γ-butyrobetaine in plasma and DBS of patients with FAODs without L-carnitine supplementation and in patients with organic acidurias (OAs) with L-carnitine supplementation and compared the data with healthy controls. At time of routine metabolite monitoring in patients, blood and plasma levels of γ-butyrobetaine were determined. First, our data suggest that plasma measurements are necessary when monitoring the carnitine status with and without carnitine supplementation. When considering plasma carnitine levels, most of the patients with FAO disorders did not present with secondary carnitine deficiency whereas levels in DBS were much lower. Second, carnitine supplementation likely inhibits carnitine biosynthesis as shown by a highly elevated γ-butyrobetaine. Interestingly, high endogenous carnitine concentrations, as observed in CPT-I deficiency, are not associated with elevated γ-butyrobetaine. Third, although free carnitine is significantly elevated in DBS in CPT-I deficiency, concentrations in plasma are much lower and diagnosis may be, therefore, missed when using plasma for confirmation diagnosis (Chapter 3; Primassin and Spiekerkoetter 2010). Overall, according to our data γ-butyrobetaine in blood can give some interesting clues, but does not serve as a reliable marker for the activation status of endogenous carnitine biosynthesis. With respect to secondary carnitine deficiency and activation of carnitine biosynthesis, a tissue analysis of carnitine biosynthesis metabolites and enzymes may be necessary (van Vlies et al. 2006).

Enzymatic assays for fatty acid oxidation

Our newly developed LC-MS/MS method for the determination of MCAD- and VLCAD-enzyme activities in human lymphocytes, as presented in chapter 4, is able to provide ample sensitivity to correctly and rapidly confirm diagnosis of the respective disorder in patients identified by NBS. As the LC-MS/MS analytical apparatus is available in all NBS laboratories worldwide, it was important to develop an analysis method compatible with available NBS instruments. This newly developed method thus offers the possibility to determine MCAD or VLCAD activity in one single setup, without exchanging chromatographic columns and lacking complex elution gradients. The assay is easy to perform in line with an assay developed for lymphocytes of patients measured by HPLC (Tajima et al. 2005). Both methods provide reference values for MCAD and VLCAD enzyme activity in lymphocytes from healthy controls that correspond well with published rates in literature. Importantly, both methods also provide ample sensitivity to discriminate not only patients from healthy individuals but, in case of MCAD deficiency, also offers the possibility to differentiate between clinical phenotypes of different severity based on residual MCAD activities, in line with their genotype (Chapter 4; ter Veld et al. 2009).

This assay was also used to measure residual enzyme activity in tissues of VLCAD KO mice to determine activity changes after provision of sufficient substrates (Chapter 5; Primassin et al. 2010). The difficulty of this assay was that enzymes in mitochondrial β-oxidation have overlapping substrate specificities and the assay actually measures the turn over rate of a special substrate and not the activity of a single enzyme. Only for a few enzymes specific substrates have been found, for example for LCAD (Wanders et al. 1998). Nevertheless this assay can be used to rapidly confirm diagnosis in individuals with a biochemical phenotype of the defect. On the basis of this and other reported methods (van Vlies et al. 2005), further assays for other enzymes of mitochondrial beta-oxidation may be developed.

Dietary modification

Currently, patients with VLCAD-deficiency are treated with a fat-reduced and fat-modified diet containing large amounts of MCT to provide sufficient energy and to avoid catabolism and tissue accumulation of long-chain acylcarnitines because of their possibly toxic effects (Spiekerkoetter et al. 2009). Only very few aliments contain MCT and therefore their ingestion with the daily diet is normally very limited. Instead, MCT has to be supplemented by special MCT product such as MCT oil or MCT margarine. Up to now

evidence-based treatment recommendations in VLCAD-deficiency are lacking (Vockley et al. 2002), however, only recently treatment recommendations based on expert opinion were released (Spiekerkoetter et al. 2009). It has been hypothesized that MCT is directly available for β-oxidation, but this has never been studied *in vivo,* and the dosage and best time of application have never been clearly defined. Case reports state that MCT preparations given just immediately prior to exercise may prevent myopathy, however, the exact underlying mechanisms have never been studied.

In the VLCAD KO mouse we could show in chapter 5 that a long-term MCT-based diet and an MCT bolus prior to exercise had significantly different effects on metabolism in liver and skeletal muscles. Continuous MCT feeding lead in VLCAD KO mice to significantly increased acylcarnitines in skeletal muscle, especially after exercise, and increased liver lipid storage. In contrast, the application of an MCT bolus prior to exercise was associated with less accumulation of long-chain acylcarnitines in skeletal muscle and less hepatic lipid storage. With MCT prior to exercise, metabolizable fatty acids were supplied during the exact time frame of increased energy demand and were able to undergo fatty acid oxidation. This study demonstrated that dietary modification with MCT in long-chain fatty acid oxidation disorders should be clearly adapted to the state of energy demand to be beneficial and to prevent myopathy. The metabolism of MCT has only partly been investigated (Bach and Babayan 1982; Marten et al. 2006), but is not yet fully understood. On the basis of our study a long-term high MCT treatment should be applied with special care (Chapter 5; Primassin et al. 2010).

In chapter 6 we additionally demonstrated that replacement of LCT with MCT, without increasing the total dietary fat content, as is common practice in the treatment of symptomatic VLCAD deficiency, resulted in severe hepatic steatosis and impaired fat metabolism. Moreover, we observed a strong up-regulation of SREBP-1c, FASN, ACC-1α and SCD1 genes clearly suggesting that the elongation and *de novo* biosynthesis of fatty acids exceeded the breakdown of MCT. The MCT-based diet had a profound impact on lipid metabolism and clearance in VLCAD KO mice, resulting in excessive accumulation of liver TGA and consequently, severe hepatic steatosis. We postulated that all the following factors: engineered ablation of VLCAD and impaired long-chain fat oxidation, up-regulation of lipogenesis, reduced ability to secrete VLDL/LDL lipoproteins and finally, enhanced hepatic fatty acid influx, act in concert and represent the underlying pathophysiological mechanism for development of serious liver disease. Thus, this unforeseen strong impairment of fat metabolism in VLCAD KO mice suggests that long-term MCT application as therapy for

human VLCAD deficiency at least calls for critical reconsideration. In contrast, the application of additional MCT prior to exercise in mice with an LCT-diet appeared to cause no steatosis and no impaired lipid metabolism, despite an overall increase in fat intake (Chapter 6; Tucci et al. 2010). A liver phenotype was also induced by fasting or cold exposure as previously shown by Spiekerkoetter et al. (Spiekerkoetter et al. 2005). Therefore, it is very important to survey the application of MCT, especially in combination with fasting events, as MCT-based diets are the mainstay of treatment (Spiekerkoetter et al. 2009).

Further recommendations for the nutritional management of these disorders include a high carbohydrate, low fat diet and avoidance of those events promoting fatty acid oxidation, such as fasting, prolonged exercise and cold exposure. We here investigated whether and how a variation of the three main components in the diet namely fat, carbohydrate and protein affect the energy status in order to avoid critical events and metabolic decompensation during increased energy demand such as during exercise. In chapter 7 we could show that the clinically normal phenotype in VLCAD KO mice was not affected by different dietary modifications under rest. However, we could also demonstrate that all dietary modifications were not able to prevent myopathy after exercise. Especially VLCAD KO mice fed with a fat-reduced diet displayed highly elevated acylcarnitines and excessive liver TGA accumulation, which gave rise to the assumption of increased hepatic lipogenesis. Nevertheless, the detection of asymptomatic VLCAD deficient patients after family screening, who did not receive a recommended fat-reduced diet at time of diagnosis also support the hypothesis that fat restriction is not the mainstay for treatment in VLCAD deficiency (Spiekerkoetter et al. 2009). In contrast, fat-enriched diets resulted in increased hepatic lipid stores and should be avoided, even though the highly fat-enriched Western diet resulted in less acylcarnitine accumulation in skeletal muscles after exercise. One hypothesis for this effect is the application of more calories since the Western diet is a high caloric diet (Chapter 7).

In conclusion, long-term treatment with various fat-modified diets could not prevent the development of myopathy after exercise. As our experiments with the MCT-bolus in chapter 5 and 6 already showed, presumably only a controlled application immediately prior to exercise can prevent these symptoms due to energy deficiency. Whether short-term energy provision may also be performed with a carbohydrate bolus needs to be further investigated.

Acylcarnitines mirror acyl-CoA content in the cells

Carnitine and acylcarnitines are assumed to mirror the acyl-CoA to free CoA ratio in the cells. To prove this hypothesis, we developed in chapter 8 a method based on ESI-MS/MS to

measure long-chain acyl-CoAs in tissues of VLCAD KO mice in parallel with the concurrent long-chain acylcarnitines. In the past, measurement of the acyl-CoA content by HPLC or ESI-MS/MS has been challenging (Minkler et al. 2008). Measurements were performed in skeletal muscle from VLCAD-deficient mice after rest and prolonged exercise in the same muscle sample and therefore, allowed accurate and direct correlation of both metabolites in sedentary and exercised muscle. We demonstrated for the first time that in skeletal muscle of VLCAD KO mouse after exercise the long-chain acylcarnitines and acyl-CoA esters both increase and directly correlate (Chapter 8; ter Veld et al. 2008). An important question is whether accumulation of acyl CoA ester or accumulation of acyclarnitines is responsible for disease pathogenesis. In HepG2 cells, incubation with C16:0-CoA and C16:0-carnitines at different concentrations demonstrated that C16:0-carnitines negatively affect cell viability and proliferation at much lower concentrations than C16:0-CoA (Chapter 2; Primassin et al. 2008). However these effects have to be proven *in vivo* in the future. Whether free CoA depletion may play an important role in disease pathogenesis also needs to be studied in the future. Further studies are underway to confirm the correlation of acyl-CoAs and acylcarnitines during carnitine supplementation.

In conclusion, all studies and results presented give further insights into disease pathophysiology in long-chain fatty acid oxidation disorders. This information is necessary to further improve treatment. Although our findings were obtained in the mouse model of VLCAD deficiency, which can obviously never allow full extrapolation to the VLCAD deficient patient, the clinical and biochemical phenotypes in both are very similar in many respects (Spiekerkoetter et al. 2005). It is therefore, conceivable and valid to suspect that comparable effects as described here may also appear in VLCAD deficient patients.

References

1. Bach AC, Babayan VK (1982) Medium-chain triglycerides: an update. Am J Clin Nutr 36:950-962

2. Exil VJ, Roberts RL, Sims H, McLaughlin JE, Malkin RA, Gardner CD, Ni G, Rottman JN, Strauss AW (2003) Very-long-chain acyl-coenzyme a dehydrogenase deficiency in mice. Circ Res 93:448-455

3. Kerner J, Hoppel C (1998) Genetic disorders of carnitine metabolism and their nutritional management. Annu Rev Nutr 18:179-206

4. Liebig M, Gyenes M, Brauers G, Ruiter JP, Wendel U, Mayatepek E, Strauss AW, Wanders RJ, Spiekerkoetter U (2006) Carnitine supplementation induces long-chain acylcarnitine production--studies in the VLCAD-deficient mouse. J Inherit Metab Dis 29:343-344

5. Marten B, Pfeuffer M, Schrezenmeir J (2006) Medium-chain triglycerides. International Dairy Journal 16:1374-1382

6. Minkler PE, Kerner J, Ingalls ST, Hoppel CL (2008) Novel isolation procedure for short-, medium-, and long-chain acyl-coenzyme A esters from tissue. Anal Biochem 376:275-276

7. Primassin S, Spiekerkoetter U (2010) ESI-MS/MS measurement of free carnitine and its precursor ã-butyrobetaine in plasma and dried blood spots from patients with organic acidurias and fatty acid oxidation disorders. Mol Genet Metab

8. Primassin S, ter Veld F, Mayatepek E, Spiekerkoetter U (2008) Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. Pediatr Res 63:632-637

9. Primassin S, Tucci S, Herebian D, Seibt A, Hoffmann L, ter Veld F, Spiekerkoetter U (2010) Pre-exercise medium-chain triglyceride application prevents acylcarnitine accumulation in skeletal muscle from very-long-chain acyl-CoA-dehydrogenase-deficient mice. J Inherit Metab Dis 33:237-246

10. Ramsay RR, Gandour RD, van der Leij FR (2001) Molecular enzymology of carnitine transfer and transport. Biochim Biophys Acta 1546:21-43

11. Spiekerkoetter U, Lindner M, Santer R, Grotzke M, Baumgartner MR, Boehles H, Das A, Haase C, Hennermann JB, Karall D, de KH, Knerr I, Koch HG, Plecko B, Roschinger W, Schwab KO, Scheible D, Wijburg FA, Zschocke J, Mayatepek E, Wendel U (2009) Treatment recommendations in long-chain fatty acid oxidation defects: consensus from a workshop. J Inherit Metab Dis 32:498-505

12. Spiekerkoetter U, Ruiter J, Tokunaga C, Wendel U, Mayatepek E, Wijburg FA, Strauss AW, Wanders RJ (2006) Evidence for impaired gluconeogenesis in very long-chain acyl-CoA dehydrogenase-deficient mice. Horm Metab Res 38:625-630

13. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Exil V, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2004) Changes in blood carnitine and acylcarnitine profiles of very long-chain acyl-CoA dehydrogenase-deficient mice subjected to stress. Eur J Clin Invest 34:191-196

14. Spiekerkoetter U, Tokunaga C, Wendel U, Mayatepek E, Ijlst L, Vaz FM, van VN, Overmars H, Duran M, Wijburg FA, Wanders RJ, Strauss AW (2005) Tissue carnitine homeostasis in very-long-chain acyl-CoA dehydrogenase-deficient mice. Pediatr Res 57:760-764

15. Tajima G, Sakura N, Yofune H, Nishimura Y, Ono H, Hasegawa Y, Hata I, Kimura M, Yamaguchi S, Shigematsu Y, Kobayashi M (2005) Enzymatic diagnosis of medium-chain acyl-CoA dehydrogenase deficiency by detecting 2-octenoyl-CoA production using high-performance liquid chromatography: a practical confirmatory test for tandem

mass spectrometry newborn screening in Japan. J Chromatogr B Analyt Technol Biomed Life Sci 823:122-130

16. ter Veld F, Mueller M, Kramer S, Haussmann U, Herebian D, Mayatepek E, Laryea MD, Primassin S, Spiekerkoetter U (2009) A novel tandem mass spectrometry method for rapid confirmation of medium- and very long-chain acyl-CoA dehydrogenase deficiency in newborns. PLoS One 4:e6449

17. ter Veld F, Primassin S, Hoffmann L, Mayatepek E, Spiekerkoetter U (2008) Corresponding increase in long-chain acyl-CoA and acylcarnitine after exercise in muscle from very long-chain acyl-CoA dehydrogenase-deficient mice. J Lipid Res

18. Tucci S, Primassin S, ter Veld F, Spiekerkoetter U (2010) Medium-chain triglycerides impair lipid metabolism and induce hepatic steatosis in very long-chain acyl-CoA dehydrogenase deficient mice. Mol Genet Metab

19. van Vlies N, Wanders RJ, Vaz FM (2006) Measurement of carnitine biosynthesis enzyme activities by tandem mass spectrometry: differences between the mouse and the rat. Anal Biochem 354:132-139

20. van Vlies N, Tian L, Overmars H, Bootsma AH, Kulik W, Wanders RJ, Wood PA, Vaz FM (2005) Characterization of carnitine and fatty acid metabolism in the long-chain acyl-CoA dehydrogenase-deficient mouse. Biochem J 387:185-193

21. Vockley J, Singh RH, Whiteman DA (2002) Diagnosis and management of defects of mitochondrial beta-oxidation. Curr Opin Clin Nutr Metab Care 5:601-609

22. Wanders RJ, Denis S, Ruiter JP, Ijlst L, Dacremont G (1998) 2,6-Dimethylheptanoyl-CoA is a specific substrate for long-chain acyl-CoA dehydrogenase (LCAD): evidence for a major role of LCAD in branched-chain fatty acid oxidation. Biochim Biophys Acta 1393:35-40

23. Winter SC (2003) Treatment of carnitine deficiency. J Inherit Metab Dis 26:171-180

Chapter 10

Summary

Fatty acid oxidation disorders (FAODs) may cause life-threatening metabolic derangements. Newborn screening (NBS) programs using tandem mass spectrometry (MS/MS) have significantly reduced disease morbidity and mortality. Very long-chain acyl-CoA dehydrogenase (VLCAD) is the first enzyme of the mitochondrial β-oxidation cycle of long-chain fatty acids. VLCAD deficiency displays heterogeneous clinical presentations due to different pathomechanisms. Various aspects of disease pathophysiology with focus on myopathy were studied with respect to carnitine homeostasis and dietary modifications.

Chapter 2 demonstrates that L-carnitine supplementation resulted in significant accumulation of potentially toxic long-chain acylcarnitines in tissues inducing myopathy. The expected prevention of secondary carnitine deficiency was not confirmed suggesting ineffectiveness of L-carnitine supplementation. Measurements in patients (chapter 3) demonstrated that L-carnitine supplementation resulted in high plasma γ-butyrobetaine which was confirmed in mouse tissues (chapter 2), thus carnitine biosynthesis was presumably inhibited. Our findings show that γ-butyrobetaine concentrations in blood as a marker for endogenous carnitine biosynthesis were not reliable, but for the carnitine status in patients, plasma should be preferred (chapter 3).

Our in chapter 4 newly developed MS/MS method is able to provide sufficient sensitivity to rapidly determine VLCAD and MCAD residual activity in human lymphocytes. Based on measured MCAD residual activities in correlation with genotype, new insights were obtained on the expected clinical phenotype. This method was also used in chapter 5 to underline the important observation that an MCT bolus directly prior to exercise may prevent the development of myopathy. In contrast, continuous long-term MCT treatment did not prevent myopathy after exercise, but stimulated hepatic lipogenesis and impaired hepatic lipid metabolism in VLCAD KO mice (chapter 6). In chapter 7 we could also show, that fat restriction did not prevent myopathic symptoms and hepatic lipid accumulation in VLCAD KO mice after exercise. Therefore fat restriction does not appear to be a mainstay of treatment in myopathic VLCAD deficiency. Sufficient energy supply appears to be essential to avoid myopathy as demonstrated for the high-caloric western type diet. The study in chapter 8 presents a direct correlation of long-chain acylcarnitines and acyl-CoAs before and after prolonged exercise in skeletal muscle of VLCAD KO mice.

In conclusion, the studies and results presented here demonstrate new insights into the pathophysiology of long-chain fatty acid oxidation defects in mice with special respect to dietary modifications and carnitine homeostasis. Moreover, these results may be a fundamental basis for the further improvement of treatment in these disorders.

Chapter 11

Zusammenfassung

Angeborene Fettstoffwechselstörungen können zu lebensbedrohlichen metabolischen Entgleisungen vor allem bei Säuglingen führen. Neugeborenenscreening unter Verwendung von Tandemmassenspektrometrie (MS/MS) hat entscheidend die Morbiditäts- und Mortalitätsraten dieser Erkrankungen verringert. Very long-chain acyl-CoA dehydrogenase (VLCAD) ist das erste Enzym des mitochondrialen β-Oxidationszyklus für langkettige Fettsäuren. VLCAD-Defizienz ist auf Grund verschiedener Pathomechanismen durch heterogene klinische Präsentation geprägt. Verschiedene Aspekte der Pathophysiologie dieser Erkrankung mit Fokus auf die Myopathie wurden in den hier durchgeführten Studien durch Analyse der Carnitinhomöostase unter verschiedenen diätetischen Modifikationen untersucht.

Kapitel 2 beschreibt wie die Supplementierung mit L-Carnitin zu einem signifikanten Anstieg von potentiell toxischen Acylcarnitinen in Geweben führte und dadurch eine Myopathie induziert wurde. Sekundäre Carnitin-Defizienz konnte nicht wie ursprünglich erwartet vermieden werden. Messungen in Patienten (Kap. 3) zeigten, dass Supplementierung mit L-Carnitin eine erhöhte Konzentration von γ-Butyrobetain zur Folge hatte und dies konnte auch in Geweben von Mäusen bestätigt werden (Kap. 2), folglich wurde die Carnitin-Biosynthese vermutlich inhibiert. Die Untersuchungen zeigten auch, dass γ-Butyrobetain im Blut nicht als Marker für endogene Carnitin-Biosynthese geeignet ist, jedoch sollte Plasma für den Carnitin-Status bei Patienten bevorzugt werden (Kap. 3).

Die in Kapitel 4 gezeigten Residualaktivitäten von VLCAD und MCAD konnten durch die von uns entwickelte MS/MS-Methode in menschlichen Lymphozyten schnell und mit ausreichender Sensitivität gezeigt werden. Basierend auf der gemessenen Residualaktivität von MCAD konnten in Korrelation mit dem Genotyp neue Einblicke bezüglich des zu erwartenden Phänotyps gewonnen werden. Diese MS/MS-Methode wurde auch in Kapitel 5 benutzt. Diese Studie zeigte, dass ein MCT Bolus direkt vor körperlicher Aktivität die Entwicklung einer Myopathie verhindern kann. Im Gegensatz dazu wird durch eine kontinuierliche Langzeittherapie mit MCT nicht nur eine Myopathie nach körperlicher Aktivität ausgelöst, sondern auch die Lipogenese stimuliert und der Lipidstoffwechsel verschlechtert (Kap. 6). In Kapitel 7 konnten wir zeigen, dass eine Fettreduktion myopathische Symptome und Lipidakkumulation in der Leber nach körperlicher Aktivität nicht verhindern konnte. Demnach scheint eine Fettreduktion nicht als Hauptstütze für die Therapie einer Myopathie geeignet zu sein. Eine ausreichende Energiebereitstellung scheint essentiell zu sein, wie eine hochkalorische Western-Diät zeigte. Die Studie in Kapitel 8 präsentiert eine direkte Korrelation zwischen langkettigen Acylcarnitinen und langkettigen Acyl-CoAs vor und nach körperlicher Aktivität im Skelettmuskel von VLCAD KO Mäusen.

Zusammenfassend geben die hier aufgezeigten Studien neue Einblicke in die Carnitin-Homöostase bei langkettigen Fettstoffwechselstörungen. Darüber hinaus können die bei der VLCAD KO Maus durch diätetische Modifikationen gewonnenen Ergebnisse als fundamentale Basis für die weitere Entwicklung von Behandlungsempfehlungen genutzt werden um klinische Phänotypen bei Patienten zu vermeiden.

Chapter 12

Publications/contributions to meetings

Primassin S, Ter Veld F, Mayatepek E, Spiekerkoetter U. Carnitine supplementation induces acylcarnitine production in tissues of very long-chain acyl-CoA dehydrogenase-deficient mice, without replenishing low free carnitine. *Pediatr Res*. 2008 Jun; 63(6):632-7.

Primassin S, Tucci S, Herebian D, Seibt A, Hoffmann L, Ter Veld F, Spiekerkoetter U. Pre-exercise medium-chain triglyceride application prevents acylcarnitine accumulation in skeletal muscle from very long-chain acyl-CoA dehydrogenase deficient-mice. *J Inherit Metab Dis*. 2010 Jun; 33(3):237- 46.

Primassin S, Spiekerkoetter U. ESI-MS/MS measurement of free carnitine and its precursor γ-butyrobetaine in plasma and dried blood spots from patients with organic acidemias and fatty acid oxidation disorders. *Mol Genet Metab*. 2010 Jun; [in press].

ter Veld F, Primassin S, Hoffmann L, Mayatepek E, Spiekerkoetter U. Corresponding increase in long-chain acyl-CoA and acylcarnitine after exercise in muscle from VLCAD mice. *J Lipid Res*. 2009 Aug; 50(8):1556-62.

ter Veld F, Mueller M, Kramer S, Haussmann U, Herebian D, Mayatepek E, Laryea MD, Primassin S, Spiekerkoetter U. A novel tandem mass spectrometry method for rapid confirmation of medium- and very long-chain acyl-CoA dehydrogenase deficiency in newborns. *PLoS One*. 2009 Jul 30;4(7):e6449.

Tucci S, Primassin S, ter Veld F, Spiekerkoetter U. Medium-chain triglycerides impair lipid metabolism and induce hepatic steatosis in very long-chain acyl-CoA dehydrogenase deficient mice. *Mol Genet Metab*. 2010 Jun; [in press].

Primaßin S, ter Veld F, Liebig M, Spiekerkötter U. Carnitinsupplementierung bei VLCAD-Mangel. APS Jahrestagung. March 2007, Fulda, Germany.

Primassin S, ter Veld F, Spiekerkoetter U. Tissue carnitine concentrations in VLCAD-deficient mice after supplementation of medium-chain fat. SSIEM. August 2007, Hamburg, Germany.

Primassin S, Tucci S, ter Veld F, Hoffmann L, Herebian D, Spiekerkoetter U. Effects of medium-chain triglycerides on fatty acid metabolism in very long-chain acyl-CoA dehydrogenase deficiency. ICIEM. August 2009, San Diego, CA, USA.

Danksagung

Diese Arbeit wäre sicher ohne die Hilfe und Unterstützung dieser Leute nicht so zustande gekommen …

In erster Linie möchte ich mich bei Ute Spiekerkötter bedanken. Sie hat mir überhaupt erst ermöglicht diese Arbeit anzufertigen und mir dieses interessante Thema bereitgestellt. Ich möchte mich für Ihre Vorschläge, Ideen und Kritiken an meinen Arbeiten wie auch für Ihren Enthusiasmus und Ihre Freundlichkeit bedanken, die das Arbeiten in Ihrer Arbeitsgruppe sehr angenehm gestaltet haben.

Bei Herrn Professor Breitkreutz bedanke ich mich für die Übernahme des Korreferats sowie das Interesse an meiner Arbeit, ohne die meine Arbeit auch nicht möglich gewesen wäre. Auch für die Hilfestellungen bezüglich des Promotionsverfahrens möchte ich mich recht herzlich bedanken.

Einen sehr großen Beitrag zum Gelingen dieser Arbeit hat Frank ter Veld geleistet durch die Übernahme der Betreuung im Labor und der vielen unzähligen Diskussionen und Anregungen sowie auch der seelischen Unterstützung und Aufbauarbeiten, wenn mal wieder nichts funktionierte. Heel erg bedankt!

Ein wichtiger Bestandteil dieser Arbeit war das Massenspektrometer. Ohne die Hilfe von Diran Herebian wäre es sicherlich nicht so viel geworden. Dafür vielen Dank! In dem Zusammenhang möchte ich mich auch bei Maurice Laryea und Gisela Schmitz für Ihre Unterstützung bedanken.

Ich bedanke mich bei den übrigen Mitgliedern der Arbeitsgruppe sowie auch des Stoffwechsellabors für die freundliche Aufnahme und angenehme Arbeitsatmosphäre. Besonders aber bei Martina Müller und bei Sara Tucci für die vielfältige Unterstützung.

Bei Ulrich Flögel und Christoph Jacoby möchte ich mich für die nette Aufnahme im Institut der Herz- und Kreislaufphysiologie von Herrn Prof. Schrader bedanken.

Und zu guter Letzt bin ich natürlich meinen Eltern, meiner Familie und Freunden sehr dankbar für die jahrelange Unterstützung und Motivation - insbesondere meinem Freund James. Cheers!