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Fasting-induced oxidative stress in very long chain acyl-CoA dehydrogenase-deficient mice

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Hepatopathy and hepatomegaly as consequences of prolonged fasting or illnesses are typical clinical features of very long chain acyl-CoA dehydrogenase (VLCACD) deficiency, the most common long-chain fatty acid β-oxidation defect. Supplementation with medium-chain triglycerides (MCTs) is an important treatment measure in these defects, in order to supply sufficient energy. Little is known about the pathogenetic mechanisms leading to hepatopathy. Here, we investigated the effects of prolonged fasting and an MCT diet on liver function. Wild-type (WT) and VLCAD knockout mice were fed with either a regular long-chain triglyceride diet or an MCT diet for 5 weeks. In both groups, we determined liver and blood lipid contents under nonfasting conditions and after 24 h of fasting. Expression of genes regulating peroxisomal and microsomal oxidation pathways was analyzed by RT-PCR. In addition, glutathione peroxidase and catalase activities, as well as thiobarbituric acid reactive substances, were examined. In VLCAD knockout mice fed with a long-chain triglyceride diet, fasting is associated with excessive accumulation of liver lipids, resulting in hepatopathy and strong upregulation of peroxisomal and microsomal oxidation pathways as well as antioxidant enzyme activities and thiobarbituric acid reactive substances. These effects were even evident in nonfasted mice fed with an MCT diet, and were particularly pronounced in fasted mice fed with an MCT diet. This study strongly suggests that liver damage in fatty acid oxidation defects is attributable to oxidative stress and generation of reactive oxygen species as a result of significant fat accumulation. An MCT diet does not prevent hepatic damage during catabolism and metabolic derangement.

Introduction

Very long chain acyl-CoA dehydrogenase (VLCAD) catalyzes the first reaction of the mitochondrial β-oxidation of long-chain fatty acids. Dysfunction and deficiency of this enzyme represents the most common β-oxidation defect of long-chain fatty acids, with an incidence of one in 55 000 to one in 100 000 births [1]. VLCAD deficiency (VLCADD) presents heterogeneous clinical phenotypes, with different severities and ages of onset, and involvement of different organ systems [2,3]. Catabolic stress or intensive physical exercise,

Abbreviations
AOX, acyl-CoA oxidase; CYP4A1, cytochrome P450 gene 4 subfamily A polypeptide 1; GPX, glutathione peroxidase; GSH, reduced glutathione; HDL, high-density lipoprotein; KO, knockout; LCT, long-chain triglyceride; LDL, low-density lipoprotein; MCT, medium-chain triglyceride; SEM, standard error of the mean; TBARS, thiobarbituric acid reactive substances; TG, triglyceride; VLCAD, very long chain acyl-CoA dehydrogenase; VLCADD, very long chain acyl-CoA dehydrogenase deficiency; VLDL, very low density lipoprotein; WT, wild type.

when energy production increasingly relies on fat metabolism, may induce or aggravate clinical symptoms and progress to severe metabolic derangement. Hypoketotic hypoglycemia, hepatomegaly, hepatopathy, Reye-like symptoms and hepatic encephalopathy are typical clinical features of prolonged fasting or of illnesses. Moreover, cardiomyopathy and skeletal myopathy also occur in long-chain fatty acid oxidation defects [4]. During these catabolic situations, long-chain fatty acids cannot be oxidized, and accumulate in tissues as long-chain acyl-CoAs and acylcarnitines [5]. However, despite the well-known mechanism of long-chain acylcarnitine accumulation, the consequences of prolonged fasting for liver lipid metabolism and liver function are poorly defined.

Medium-chain triglycerides (MCTs) have been reported to bypass the first step of β-oxidation catalyzed by VLCAD, and can be fully metabolized [6,7]. Therefore, treatment recommendations for VLCADD include avoidance of fasting, and a long-chain triglyceride (LCT)-restricted and fat-modified diet, in which LCTs are completely or in part replaced by MCTs [7–9]. Supplementation with MCTs has been proven to be especially effective in cardiac and myopathic phenotypes [10].

The effects of dietary intervention in VLCADD can be easily studied with the VLCAD knockout (KO) mouse model, that has similar clinical symptoms to those observed in human VLCADD [5]. In fact, in both mice and humans, clinical symptoms become mainly evident as a consequence of triggers such as fasting, resulting in the accumulation of long-chain acylcarnitines, hypoglycemia, and hepatopathy [1].

The pathophysiology behind the hepatic damage is not well understood. Oxidative stress has often been discussed, but has never been proven. To gain insights into the pathogenetic mechanisms involved in the development of hepatopathy and hepatomegaly, we studied wild-type (WT) and VLCAD KO mice fed with either a normal LCT diet or a long-term MCT diet. To study hepatic effects during anabolism and catabolism, analyses were carried out under regular feeding and after 24 h of fasting with and without dietary intervention. We measured liver and blood lipid concentrations as well as the expression at the mRNA level of acyl-CoA oxidase (*AOX*) and cytochrome P450 gene 4 subfamily A polypeptide 1 (*CYP4A1*), which are involved in peroxisomal and microsomal fatty acid oxidation, respectively. Moreover, we measured the activity of antioxidant enzymes, as well as the concentration of thiobarbituric acid reactive substances (TBARS) resulting from decomposition of lipid peroxide products.

Table 1. Ratio liver/body weight in WT and VLCAD KO mice.

Diet	WT	VLCAD KO
LCT		
Nonfasted	0.39 ± 0.01[a]	0.45 ± 0.01[a,b]
Fasted	0.50 ± 0.02[c]	0.66 ± 0.01[b,c]
MCT		
Nonfasted	0.5 ± 0.03[a,d]	0.52 ± 0.02[a,d]
Fasted	0.42 ± 0.02[c,d]	0.52 ± 0.01[d]

[a] Values obtained by Tucci *et al.* [13]. [b] Significant differences between WT and VLCAD KO mice within a group. [c] Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen. [d] Significant differences between WT and VLCAD KO mice under different dietary conditions.

Results

Clinical phenotype

Fasting resulted in both genotypes fed with an LCT diet having significantly higher liver/body weight ratios. As an effect of an MCT diet, WT and VLCAD KO mice displayed higher liver/body weight ratios under nonfasting conditions (Table 1). Moreover, the MCT diet and fasting resulted in significantly lower liver/body weight ratios in both WT and VLCAD KO mice than the LCT diet and fasting.

Intrahepatic lipid content

As VLCAD KO mice cannot oxidize long-chain fatty acids during catabolic situations, we tested the accumulation of liver lipids after 24 h of fasting. Under an LCT diet, VLCAD KO mice displayed significantly higher intrahepatic lipid accumulation, 39.4 ± 4.7% of the dry weight, whereas no difference was observed in WT mice. In contrast, both genotypes fed with the MCT diet already displayed significantly higher liver lipids – 21.4 ± 1.6% of the dry weight in the WT mice and 26.4 ± 3.1% in the VLCAD KO mice – under nonfasting conditions, and these percentages increased further with fasting (Fig. 1).

In parallel with liver lipids, liver triglyceride (TG) content significantly increased after fasting, with both an LCT and an MCT diet. It is concerning that an MCT diet alone without fasting also induced further lipid accumulation (Fig. 1).

Blood lipid profile

VLCAD KO mice had significantly higher total cholesterol than WT mice. With an MCT diet, total serum

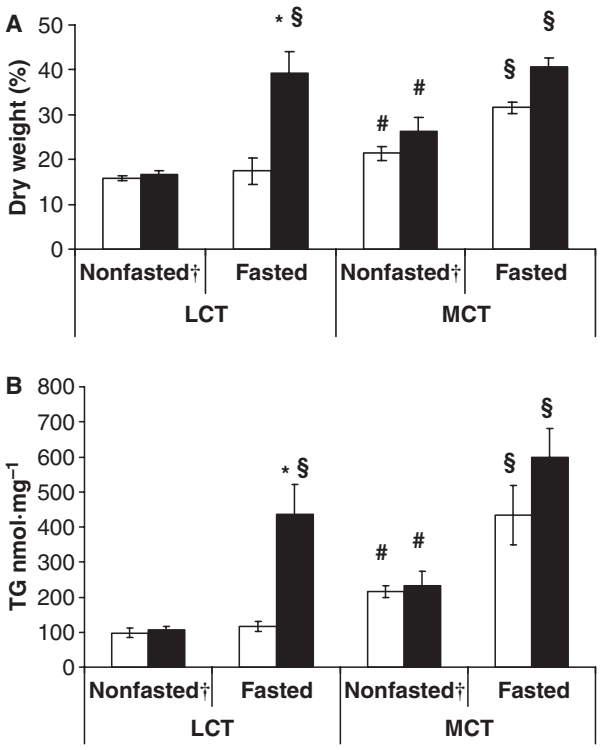

Fig. 1. Intrahepatic (A) lipid content and (B) TG content. Mean concentrations are given. The values are mean ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice under nonfasting conditions and after 24 h of fasting. White bars and black bars represent WT and VLCAD KO mice, respectively. Values were considered to be significant if $P < 0.05$. *Significant differences between WT and VLCAD KO mice within a group. #Significant differences between WT and VLCAD KO mice under different dietary conditions. §Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen. †Values obtained by Tucci *et al.* [13].

cholesterol was even higher in VLCAD KO mice. After fasting, as expected, total cholesterol significantly decreased with both diets (Fig. 2A). Importantly, fasting significantly increased the very low density lipoprotein (VLDL)/low-density lipoprotein (LDL) cholesterol ratio in VLCAD KO mice on the LCT diet, but not in those on the MCT diet (Fig. 2B). High-density lipoprotein (HDL) cholesterol was mainly regulated by the feeding state, and was significantly increased by fasting (Fig. 2C).

RT-PCR and gene expression

Because of the hepatic lipid accumulation after fasting [11], we tested the expression at the mRNA level of two genes involved in alternative oxidation pathways, those encoding peroxisomal AOX and the microsomal CYP4A1 hydroxylase. RT-PCR analysis revealed that with an LCT diet and no fasting, the expression of *AOX* was significantly higher in VLCAD KO mice than in WT mice. Fasting induced a significant increase in both genotypes; however, this was more evident in the VLCAD KO mice (Fig. 3A). Interestingly, the MCT diet also induced *AOX* gene expression in WT mice. After 24 h of fasting, both genotypes showed a significant increase in the expression of *AOX* with the MCT diet. As shown in Fig. 3B, under nonfasting conditions, the expression of *CYP4A1* was higher in VLCAD KO mice than in WT mice under both dietary regimens, although the difference was not significant, and was upregulated after fasting. With an MCT diet and after fasting, the expression of *CYP4A1* was particularly high.

Liver oxidative stress

Glutathione peroxidase (GPX)

The activity of GPX did not differ between WT and VLCAD KO mice fed with an LCT diet, when mice were not fasted. However, the activity significantly increased from 53.56 ± 5.3 to 78.58 ± 5.5 U·mg^{-1} in WT mice and from 48.29 ± 5.2 to 147.43 ± 20.4 U·mg^{-1} in VLCAD KO mice after fasting (Fig. 4). Of concern was the fact that the MCT diet increased GPX activity to 70.95 ± 4.4 U·mg^{-1} in WT mice and 91.55 ± 8.5 U·mg^{-1} in VLCAD KO mice in the nonfasted state. Interestingly, the MCT diet combined with fasting significantly reduced GPX activity in WT mice, from 70.95 ± 4.4 to 47.56 ± 9.4 U·mg^{-1}, whereas it remained high in VLCAD KO mice.

Reduced glutathione (GSH)

GSH is the substrate for GPX, so we quantified GSH under both dietary conditions. Both genotypes fed with the LCT diet showed no differences in GSH content when not fasted. However, we observed a direct correlation between increased GPX activity and significant reduction in GSH amount after fasting in VLCAD KO mice (Fig. 4). This fasting effect was also observed with the MCT diet, with a GSH value of 32.82 ± 2.0 nmol·mg^{-1} that decreased to 22.58 ± 1.2 nmol·mg^{-1} in WT mice, and a GSH value of 30.53 ± 1.5 nmol·mg^{-1} that decreased to 21.02 ± 1.3 nmol·mg^{-1} in VLCAD KO mice.

Catalase activity

Similar results were obtained for catalase activity, as shown in Fig. 5. With LCT and fasting, catalase activity significantly increased up to 320.4 ± 17.8 and 515.8 ± 20.7 U·mg^{-1} in WT and VLCAD KO mice,

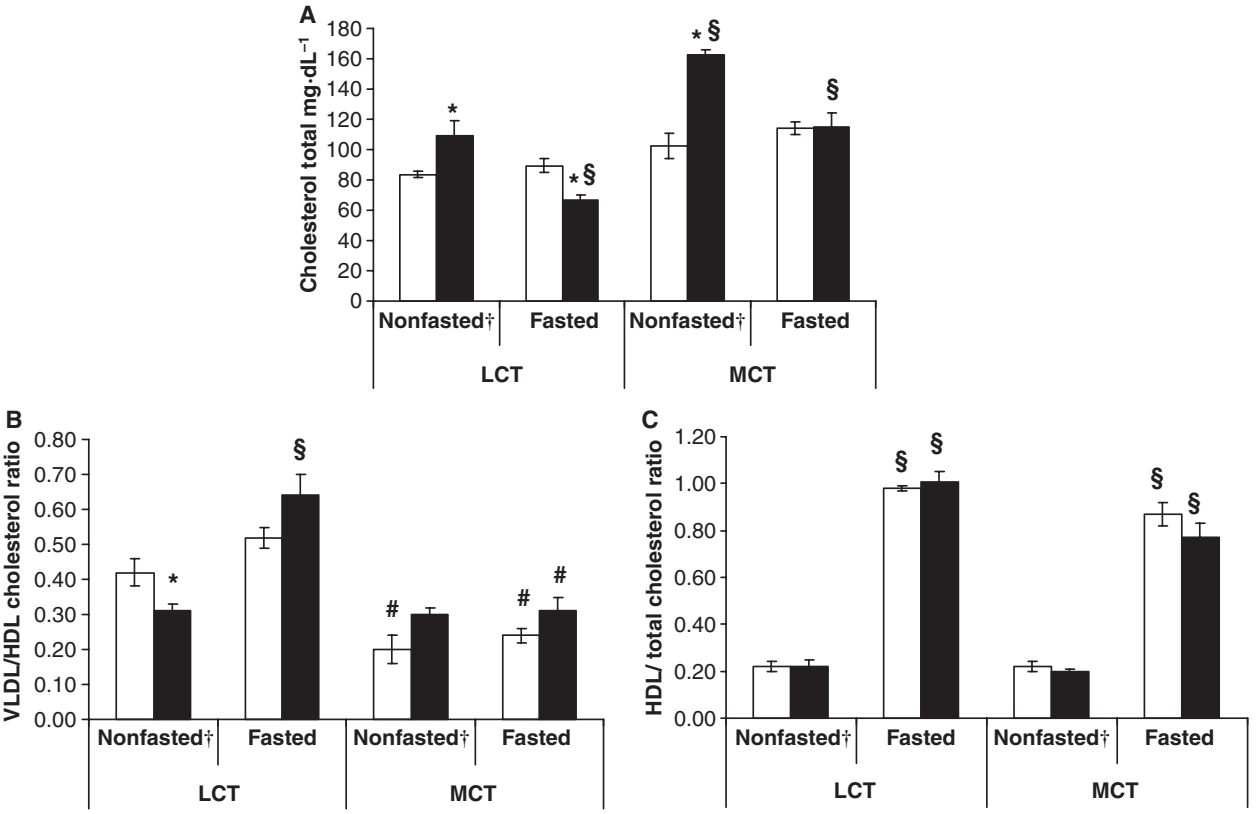

Fig. 2. Cholesterol in serum samples of WT and VLCAD KO mice. Mean concentrations are given. The values are mean ± SEM for WT (n = 5) and VLCAD KO (n = 5) mice per dietary group under nonfasting conditions and after 24 h of fasting. White bars and black bars represent WT and VLCAD KO mice, respectively. Values were considered to be significant if $P < 0.05$. *Significant differences between WT and VLCAD KO mice within a group. #Significant differences between WT and VLCAD KO mice under different dietary conditions. §Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen. †Values obtained by Tucci *et al.* [13].

respectively. VLCAD KO mice fed with the MCT diet presented significantly higher catalase activity in the nonfasting state than VLCAD KO mice fed with the LCT diet. Fasting further increased catalase activity in the MCT-fed mice.

TBARS

As shown in Fig. 5, VLCAD KO mice fed with the LCT diet in displayed, in the nonfasted state, a nearly four-fold higher TBARS concentration than WT mice. Fasting induced further TBARS production. Surprisingly, both genotypes fed with the MCT diet showed, when nonfasted, very similar TBARS concentrations as those in fasted mice fed with the LCT diet. The TBARS content in fasted mice fed with the MCT diet, however, directly correlated with GPX activity, in that TBARS content decreased in WT mice, whereas it rose significantly from 140.72 ± 23.3 to 230.98 ± 13.78 nmol·mg^{-1} in VLCAD KO mice.

Discussion

The present study provides strong evidence that fasting-induced hepatopathy and hepatomegaly are closely related to the development of oxidative stress in VLCAD KO mice. An important observation is that MCT provides sufficient energy for skeletal and cardiac muscles to prevent or reverse cardiomyopathy or skeletal myopathy [10]; however, it does not prevent hepatopathy during catabolic situations. In fact, we observed a marked upregulation of *AOX* and *CYP4A1* with the MCT diet, resulting in a constitutive increment of reactive oxygen species (ROS), which may be associated with a substantial risk of ROS-induced liver damage.

Fasting is characterized by a considerable influx of fatty acids into the liver. As a consequence, the β-oxidation rate is increased [12]. However, as VLCAD KO mice are unable to oxidize long-chain fatty acids, liver lipid accumulation after fasting is particularly evident.

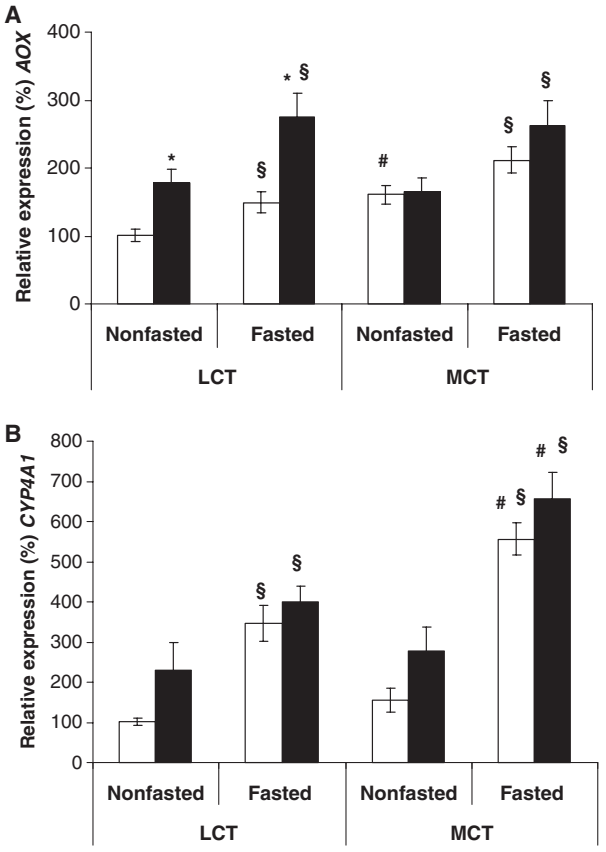

Fig. 3. Relative expression of *AOX* (A) and *CYP4A1* (B) genes at the mRNA level. The values are mean ± SEM for WT ($n = 5$) and VLCAD KO ($n = 5$) mice per dietary group under nonfasting conditions and after 24 h of fasting. White bars and black bars represent WT and VLCAD KO mice, respectively. Values were considered to be significant if $P < 0.05$. *Significant differences between WT and VLCAD KO mice within a group. #Significant differences between WT and VLCAD KO mice under different dietary conditions. §Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen.

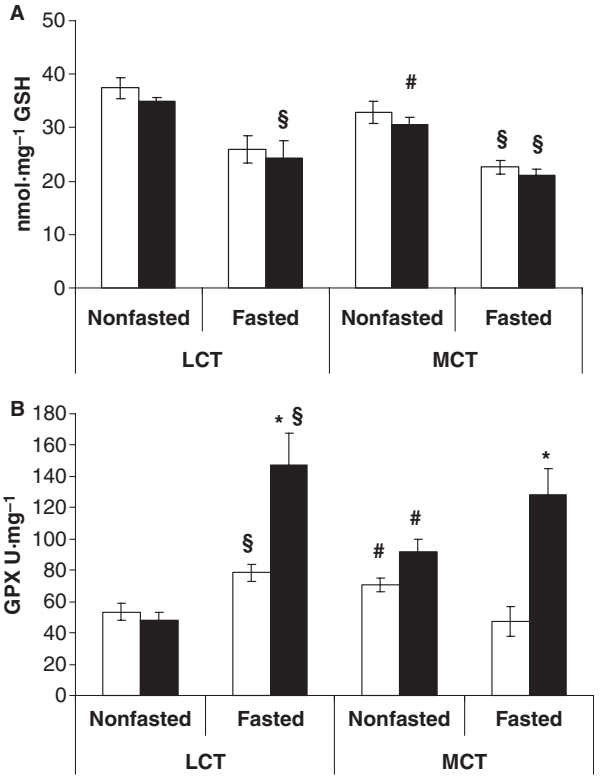

Fig. 4. GPX (A) and GSH (B) in liver of WT and VLCAD KO mice. Mean concentrations are given. The values are mean ± SEM for WT ($n = 5$) and VLCAD KO ($n = 5$) mice per dietary group under nonfasting conditions and after 24 h of fasting. White bars and black bars represent WT and VLCAD KO mice, respectively. Values were considered to be significant if $P < 0.05$. *Significant differences between WT and VLCAD KO mice within a group. #Significant differences between WT and VLCAD KO mice under different dietary conditions. §Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen.

The parallel increases in liver TGs and liver/body weight ratio confirm the inability of the liver to perform β-oxidation of fatty acids, which therefore accumulate. Importantly, lipid and TG accumulation occurred in the same proportions in fasted mice previously fed with the MCT diet. In fact, with the MCT diet, lipid and TG accumulation was evident not only in VLCAD KO mice but also in WT mice. These data confirm impaired lipid metabolism and clearance with high MCT amounts, even without an underlying mitochondrial β-oxidation defect [13].

In line with other studies [14,15], we observed that the cholesterol concentrations in VLCAD KO mice under both dietary regimens were increased, and only decreased after fasting, as expected, suggesting the need for careful monitoring of fat metabolism in patients with fatty acid oxidation defects. In addition, the increased VLDL/LDL cholesterol ratio in fasted VLCAD KO mice fed with the LCT diet shows that the fasting-induced liver lipid accumulation is associated with impaired assembly and secretion of VLDL. Overall, there is increasing evidence that an inherited enzyme defect in mitochondrial β-oxidation also affects many other pathways of lipid metabolism [13].

The transcription of genes related to mitochondrial and peroxisomal oxidation is an adaptive response to fasting. As peroxisome proliferator-activated receptor-α is responsible for the management of energy stores during fasting [16–18], the peroxisome proliferator-activated receptor-α-dependent pathways, including *CYP4A1*, are upregulated. Our results confirmed that,

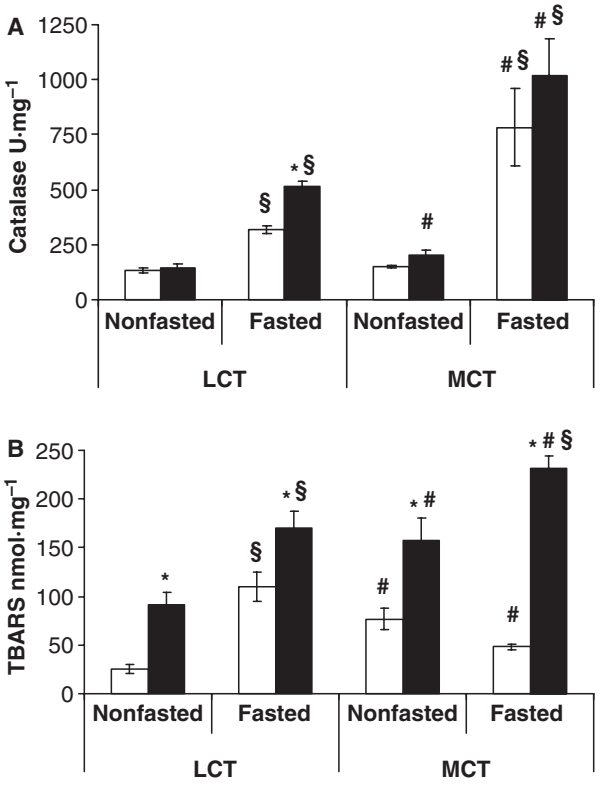

Fig. 5. Catalase activity (A) and TBARS (B) in liver of WT and VLCAD KO mice. Mean concentrations are given. The values are mean ± SEM for WT ($n = 5$) and VLCAD KO ($n = 5$) mice per dietary group under nonfasting conditions and after 24 h of fasting. White bars and black bars represent WT and VLCAD KO mice, respectively. Values were considered to be significant if $P < 0.05$. *Significant differences between WT and VLCAD KO mice within a group. #Significant differences between WT and VLCAD KO mice under different dietary conditions. §Significant differences between WT and VLCAD KO mice under nonfasting and fasting conditions within the same dietary regimen.

after fasting, *AOX* expression was strongly upregulated in both genotypes with an LCT diet, in agreement with previous results [19]. However, mice fed with the MCT diet displayed upregulation of *AOX* expression at the mRNA level in the nonfasted state, and a further increase after fasting. Very similar results were obtained for the expression of *CYP4A1*, with a significant induction of *CYP4A1* gene expression in fasted mice previously fed with the LCT diet. Despite the pivotal role of CYP4A1 in lipid oxidation and the provision of nutrients needed for peripheral tissues, CYP4A1 increases the synthesis of dicarboxylic and ω-hydroxylated fatty acids, which may impair mitochondrial oxidative phosphorylation [20,21]. Although both alternative fatty acid oxidation pathways are efficient systems for the removal of excessive cytosolic free fatty acids and their toxic derivatives, they generate ROS, inducing oxidative stress [22,23]. The association between the upregulation of microsomal/peroxisomal pathways and the development of steatohepatitis resulting from increased production of ROS have been described previously [24–26], as has the correlation of antioxidant enzyme activity with lipid peroxidation in different human diseases [27–30]. Fasted VLCAD KO mice fed with the LCT diet displayed much higher GPX activity than nonfasted VLCAD KO mice. As GPX is responsible for detoxification of mitochondrial hydrogen peroxides [31], our results suggest that the electron flow through the respiratory chain is partially hampered by the excessive fasting-induced accumulation of liver lipids that cannot be oxidized and processed. Increased GPX activity, together with a reduced GSH content and increased liver lipid accumulation, was also observed in nonfasted VLCAD KO mice fed with the MCT diet. These data support our hypothesis that high amounts of MCTs aggravate hepatic damage. Further evidence is the significant increase in catalase activity observed after fasting in mice fed with the MCT diet. Catalase is localized in peroxisomes, and traps hydrogen peroxides arising during the oxidation of fatty acids catalyzed by AOX, detoxifying them to water and oxygen. Moreover, previous studies [13,32] have demonstrated that an MCT diet stimulates lipogenesis and raises the concentration in plasma of long-chain fatty acids, which are the preferred substrates for peroxisomal β-oxidation [33,34].

In addition to the direct mechanisms of fatty acid toxicity resulting from excessive intracellular accumulation, lipid peroxidation also plays a key role involving polyunsaturated fatty acids in either the free or esterified state. In fact, ROS can react with cellular fatty acids, initiating the autopropagative processing of lipid peroxides that are potentially toxic for tissues [35]. We show here that in VLCAD KO mice fed with the LCT diet, the concentration of TBARS was three-fold higher than in WT mice, suggesting chronic activation of the peroxisomal pathway to compensate for deficient mitochondrial β-oxidation. The increased TBARS concentration in mice fed with the MCT diet mirrors the effects observed for GPX and catalase activities, thus indicating that a diet based on MCTs raises the risk of ROS production. The TBARS concentration was strongly increased after fasting under both dietary regimens, as an indirect consequence of enhanced fatty acid influx into the liver. These data underline the fact that hepatopathy during fasting can most likely be ascribed to ROS-dependent effects. VLCAD KO mice show signs of oxidative stress under nonfasting conditions and with the LCT diet. However, this effect was

more pronounced in VLCAD KO mice fed with the MCT diet.

In summary, this study demonstrates that, in VLCAD KO mice, fasting is associated with excessive accumulation of liver lipids, resulting in hepatopathy and strong upregulation of peroxisomal and microsomal oxidation pathways. As a consequence, the generation of ROS and lipid peroxides is induced. Importantly, supplementation with MCTs does not prevent these effects. In fact, high amounts of MCTs aggravate ROS production and oxidative stress. Given the effects of an MCT diet, we suppose that in medium-chain acyl-CoA dehydrogenase deficiency during metabolic derangement with accumulation of medium-chain fatty acids, the same mechanism of upregulation of peroxisomal and microsomal oxidation pathways may be responsible for acute liver dysfunction. In conclusion, whereas MCT supplementation significantly improves cardiac and skeletal muscle symptoms in fatty acid oxidation defects resulting from energy deficiency, its use with respect to the hepatic phenotype of VLCAD deficiency has to be carefully considered and closely monitored.

Experimental procedures

Reagents

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

Animals

The VLCAD KO mice used in these studies were kindly provided by A. W. Strauss (currently Cincinnati Children's Hospital, OH, USA), and were generated as described in detail previously [36]. Experiments were performed on fourth-generation to fifth-generation intercrosses of C57BL6 + 129sv VLCAD genotypes. Littermates served as controls, and genotyping of mice was performed as previously described [36].

Groups consisting of five mice, 10–12 weeks of age, were investigated under well-fed, nonfasting conditions. Mice were killed by CO_2 asphyxiation. 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 killed either immediately or after 24 h of fasting. Livers were 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

After weaning, at approximately 5–6 weeks of age, mice were divided into two groups and fed with different diets for 5 weeks. The first group received a purified mouse diet containing 5.1% crude fat in the form of LCTs, corresponding to 13% of metabolizable energy, as 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 Spezialdiäten GmbH) that also corresponding to ∼ 13% of total metabolizable energy as calculated with Atwater factors, in which 4.4% from a total amount of 5% fat comprised MCTs (Ceres MCT-oil; basis GmbH, Oberpfaffenhofen, Germany), and the remaining 0.6% was derived from the soy bean oil, to provide the required long-chain essential fatty acids. In both diets, carbohydrate and protein concentrations were unmodified, and corresponded to 65% and 22% of metabolizable energy, respectively. Mice received water *ad libitum* during both well-fed and fasting conditions.

Lipid and lipoprotein analysis

Lipoprotein concentrations were measured in duplicate in serum samples by using enzymatic kits (EnzyChrom HDL and VLDL/LDL Assay kit; BioTrend, Cologne, Germany) on an Infinite M200 Tecan (Crailsheim, Germany) plate reader. Liver TGs were measured in duplicate by using enzymatic kits (EnzyChrom Triglyceride Assay kit; BioTrend). All assays were performed according to the manufacturer's instructions.

Intrahepatic lipid content

The intrahepatic lipid content was measured gravimetrically according to the method of Folch *et al.* [37], modified as previously reported [13].

Liver homogenates and enzyme activities

Liver was homogenized in one volume of ice-cold $NaCl/P_i$ (pH 7.3), and then centrifuged at 16 000 ***g*** for 15 min at 4 °C to remove any cell debris. The clear supernatant was used immediately for the enzyme assays or stored at −80 °C. The protein concentration of tissue homogenates was determined with the method of Bradford, as described previously [38].

GSH was measured in liver homogenates by using an enzymatic kit (Glutathione Assay kit; Bio Trend). Catalase activity was measured fluorometrically by the production of the highly fluorescent oxidation product resorufin [39,40].

Table 2. Forward and reverse primers used for RT-PCR analysis.

	Forward (5′–3′)	Reverse (5′–3′)
β-Actin	TAGGCACCAGGGTGTGATGG	CTCCATGTCGTCCCAGTTGG
AOX	TGCCCAGGTGAGAAGCCTGACG	TCAGACTGGCGCCTCACAGC
CYP4A1	CTCATTCCTGCCCTTCTCAG	TCCCATTTTTGGACTTCAGC

GPX activity was determined by calculating the rate of oxidation of NADPH to $NADP^+$ spectrophotometrically at 340 nm for 4 min, as previously described [41,42]. The concentration of TBARSs resulting from decomposition of lipid peroxide products was determined fluorimetrically as previously described [43].

RT-PCR analysis

Total liver RNA was isolated with the RNeasy mini kit (Qiagen, Hilden, Germany). Forward and reverse primers for β-actin (BC138614), AOX (NM_015729.2) and CYP4A1 (NM_010011.3) were designed with the FASTPCR program (R. Kalendar, Institute of Biotechnology, Helsinki), and are shown in Table 2. RT-PCR was performed in a single-step procedure with the QuantiTect SYBR Green RT-PCR (Qiagen) on an Applied Biosystems 7900HT 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 [44]. 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 errors of the mean (SEMs), with *n* denoting the number of animals tested. Analysis for the significance of differences was performed with Student's *t*-tests for paired and unpaired data. Two-way ANOVA with Bonferroni *post hoc* tests was performed with GRAPHPAD PRISM (GraphPad Software, San Diego, CA, USA). Differences were considered to be significant if $P < 0.05$.

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