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Sexual dimorphism of lipid metabolism in very long-chain acyl-CoA dehydrogenase deficient (VLCAD$^{-/-}$) mice in response to medium-chain triglycerides (MCT)

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ABSTRACT

Medium-chain triglycerides (MCT) are widely applied in the treatment of long-chain fatty acid oxidation disorders. Previously it was shown that long-term MCT supplementation strongly affects lipid metabolism in mice. We here investigate sex-specific effects in mice with very-long-chain-acyl-CoA dehydrogenase (VLCAD) deficiency in response to a long-term MCT modified diet. We quantified blood lipids, acylcarnitines, glucose, insulin and free fatty acids, as well as tissue triglycerides in the liver and skeletal muscle under a control and an MCT diet over 1 year. In addition, visceral and hepatic fat content and muscular intramyocellular lipids (IMCL) were assessed by *in vivo* ^{1}H MRS techniques. The long-term application of an MCT diet induced a marked alteration of glucose homeostasis. However, only VLCAD$^{-/-}$ female mice developed a severe metabolic syndrome characterized by marked insulin resistance, dyslipidemia, severe hepatic and visceral steatosis, whereas VLCAD$^{-/-}$ males seemed to be protected and only presented with milder insulin resistance. Moreover, the highly saturated MCT diet is associated with a decreased hepatic stearoyl-CoA desaturase 1 (SCD1) activity in females aggravating the harmful effects of a saturated MCT diet. Long-term MCT supplementation deeply affects lipid metabolism in a sexual dimorphic manner resulting in a severe metabolic syndrome only in female mice. These findings are striking since the first signs of insulin resistance already occur in female VLCAD$^{-/-}$ mice during their reproductive period. How these metabolic adaptations are finally regulated needs to be determined. More important, the relevance of these findings for humans under these dietary modifications needs to be investigated.

1. Introduction

Lipid metabolism is differently regulated between the sexes resulting in various phenotypes with regard to body composition, body fat distribution and substrate metabolism. It is known that lipid metabolism and expression of genes coding for fatty acid oxidation (FAO) enzymes are regulated by different means such as dietary fatty acids [1] or the transcription factor peroxisome proliferator-activated receptor α (*PPARα*). In addition, sex hormones do play an important role and determine regulation in a sexually dimorphic manner [2,3]. Therefore, the degree of disturbance in fatty acid metabolism and lipid homeostasis as they occur in inherited fatty acid oxidation disorders (FAOD) may also be strongly gender-specific.

FAOD are a group of diseases comprising defective enzymes of the mitochondrial β-oxidation [4]. Symptoms occur mainly during catabolic situations such as prolonged fasting or illnesses presenting with hypoketotic hypoglycemia, hepatic encephalopathy, cardiomyopathy and skeletal myopathy [5]. Very long-chain acyl-CoA dehydrogenase deficiency (VLCADD) is considered the most common defect of the mitochondrial oxidation of long-chain fatty acids with an incidence of 1:50,000–1:100,000 [5–7]. VLCADD is characterized by different clinical phenotypes as well as severity and age at onset [8,9]. The recommended therapeutical approach includes the replacement of long-chain triglycerides by medium-chain triaglycerides (MCT), which can be fully oxidized in the mitochondrial β-oxidation. The clinical efficacy of MCT is widely recognized especially with respect to the prevention and treatment of cardiomyopathy and muscular symptoms [10,11]. Although an MCT diet is considered a safe dietary intervention and is applied in different FAOD for longer periods of time, recent reports highlight the adverse effects of an MCT diet in the murine model of VLCADD [12–16]. Long-term supplementation over one year contributed to the development of an unexpected clinical phenotype with an increased body fat content and a disturbance in body fat composition in the mouse model of VLCADD [13,16]. Because of these significant changes due to dietary interventions in the VLCAD mouse, we here assessed possible additional sex-specific effects.

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Biochemical parameters such as blood lipids, glucose, insulin and free fatty acids were measured, while the concentration of triglycerides (TAGs) was assessed in the liver and skeletal muscle of WT and VLCAD$^{-/-}$ mice under a control diet and after MCT supplementation. Acylcarnitine profiles were analyzed in dried blood spots to evaluate the metabolic state of WT and VLCAD$^{-/-}$ mice. Visceral and hepatic fat as well as the muscular IMCL content were assessed by *in vivo* ^{1}H (MR) techniques.

2. Materials and methods

2.1. Animals

Experiments were performed on the fourth- to fifth-generation intercrosses of C57BL6 + 129sv VLCAD genotypes. Littermates served as controls and genotyping of mice was performed as described previously in Exil et al. [17]. Serum parameters were determined under standard conditions and blood was taken 5 h after food intake at the age of six months. Mice at the age of 12 months were sacrificed immediately after MR investigation by CO_2 asphyxiation.

Blood samples were collected by heart puncture. Serum was obtained by centrifugation at 16,000 *g* for 10 min and stored at −80 °C for further analysis. The liver and skeletal muscle were rapidly removed and immediately frozen in liquid nitrogen. The right anterior and medial lobes of the liver with the gall bladder were transferred in 10% formaldehyde for histopathology.

All animal studies were performed with the approval of the University's Institutional Animal Care and Use Committee and in accordance with the Committees' (LANUV) guidelines.

2.2. Diet composition and supplementation

At 5 weeks of age, mice of each genotype were divided in two groups and fed with different diets for one year. The first group received a normal purified mouse diet containing 5% crude fat in the form of LCT, corresponding to 12% 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 corresponding as well to 12% of total metabolizable energy. Here, 4.4% from a total of 5% fat was MCT (Ceres®MCT-oil, basis GmbH, Oberpfaffenhofen, Germany) while the remaining 0.6% was derived from soybean oil providing the required essential long-chain fatty acids. The necessary amount of essential long-chain fatty acids was calculated in accordance to the Nutrient Requirements of Laboratory Animals (Subcommittee on Laboratory Animal Nutrition, Committee on Animal Nutrition, Board on Agriculture, National Research Council). Both diets based on purified feed ingredients contained the same nutrient concentration as follows: 94.8% dry matter, 17.8% crude protein (N × 6.25), 5% crude fat, 5% crude fiber, 5.3% crude ash, 61.9% nitrogen free extract, 36.8% starch, 14.8% dextrin and 11% sugar. The detailed fatty acid composition of the diets was previously reported [13]. In both diets the carbohydrate and protein contents corresponded to 69% and 19% of metabolizable energy, respectively. All mouse groups received water *ad libitum*.

2.3. Magnetic resonance imaging (MRI)

2.3.1. General setup

Data were recorded on a Bruker Avance^{III} 9.4 Tesla Wide Bore (89 mm) nuclear magnetic resonance (MR) spectrometer operating at frequencies of 400.13 MHz for ^{1}H as previously described [13,18].

2.3.2. Intra- and extramyocellular lipids (IMCL and EMCL)

The mice were positioned on their left side within the animal handling system and the right hind leg was fixed in a 10-mm saddle coil with the *Tibialis anterior* (TA) muscle aligned along the main magnetic field direction ensuring maximal spectral separation of IMCL and EMCL resonances [19]. Image acquisition and IMCL quantification have been performed as previously described [20].

2.4. Histological evaluation

Liver tissue was excised from the eviscerated animals and fixed in 10% formalin. For light microscopy examination, the tissues were embedded in paraffin and sectioned at 5 μm. Liver slices of all analyzed mice were evaluated with a magnification of ×33 and stained with hematoxylin and eosin (H&E) for assessment of steatosis, inflammation, and necrosis or Sirius red for assessment of fibrosis. To determine lipid content, 10 μm thick cryostat sections were collected on Superfrost slides, and stained with Sudan III. Steatosis, degree of inflammation and stage of fibrosis were assessed as previously described [13,21].

2.5. Liver homogenates, triglyceride (TAG) and lipid peroxide content

Tissues were homogenized in CelLytic MT Buffer (Sigma-Aldrich, Steinheim, Germany) in the presence of 1 mg·mL^{-1} protease inhibitors and centrifuged at 4 °C and 16,000 *g* for 10 min to pelletize any cell debris. The clear supernatant was immediately used for the enzyme assays or stored at −80 °C.

The concentration of thiobarbituric acid reactive substances (TBARS) resulting from decomposition of lipid peroxide products was determined fluorimetrically in serum and liver tissue as previously described [14]. TAG concentrations were measured in the liver as duplicates by using enzymatic kits (EnzyChrom triacylglyceride Assay Kit, BioTrend, Cologne, Germany) following the manufacturer's instructions. Briefly, TAGs were extracted from the liver and the concentration was measured enzymatically. Extracted TAGs were hydrolyzed to free fatty acids and glycerol. This reacted with a dye to generate a colored compound visible at 570 nm (spectrophotometry) or at Ex/Em = 535/587 nm (fluorescence).

2.6. Analysis of serum variables and transaminases

Free fatty acid (FFA), TAG and lipoprotein concentrations were measured as duplicates in serum samples as described previously [15]. Glucose and ketone bodies were determined with a Precision Xceed blood sugar meter (Abbott, Wiesbaden, Germany).

Insulin was measured in duplicate by using the Ultrasensitive Mouse Insulin ELISA Kit (Mercodia AB, Uppsala Sweden). Oxidized low density lipoproteins (ox-LDL) were quantified in duplicates by using the Enzyme-linked Immunosorbent Assay Kit for ox-LDL (Hölzel Diagnostika, Cologne, Germany). Aspartate aminotransferase (AST) and alanine aminotransferase (ALT) were determined at 37 °C accordingly to the ICFF procedures [22,23]. Insulin resistance was calculated by the homeostasis monitoring assessment (HOMA) formula [24]. The homeostasis model assessment (HOMA) of insulin resistance index, as described by Matthews et al. [24], is the most easily obtained measurement of insulin resistance which can be used as a reliable surrogate measure of *in vivo* insulin sensitivity since this method correctly differentiated between insulin sensitivity and insulin resistance [25]. HOMA index was calculated with glucose and insulin concentrations obtained after 5 h of fasting using the following formula: fasting blood glucose (md/dL) × fasting insulin (μU/mL) / 22.5.

2.7. Analysis of acylcarnitines

Analysis of acylcarnitines was performed as described previously [26–28]. Briefly, acylcarnitines were extracted from dried blood spots and tissues with acetonitrile/water (80/20% v/v) in the presence of [$^{2}H_3$] free carnitine, [$^{2}H_3$] octanoyl-carnitine and [$^{2}H_3$] palmitoyl-carnitine as internal standards. The extracted supernatant was dried and the butylated acylcarnitines were analyzed by electron spray

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