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# Dietary eicosapentaenoic acid supplementation accentuates hepatic triglyceride accumulation in mice with impaired fatty acid oxidation capacity

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ABSTRACT

Reduced mitochondrial fatty acid (FA) β-oxidation can cause accumulation of triglyceride in liver, while intake of eicosapentaenoic acid (EPA) has been recommended as a promising novel therapy to decrease hepatic triglyceride content. However, reduced mitochondrial FA β-oxidation also facilitates accumulation of EPA. To investigate the interplay between EPA administration, mitochondrial activity and hepatic triglyceride accumulation, we investigated the effects of EPA administration to carnitine-deficient mice with impaired mitochondrial FA β-oxidation. C57BL/6J mice received a high-fat diet supplemented or not with 3% EPA in the presence or absence of 500 mg mildronate/kg/day for 10 days. Liver mitochondrial and peroxisomal oxidation, lipid classes and FA composition were determined. Histological staining was performed and mRNA level of genes related to lipid metabolism and inflammation in liver and adipose tissue was determined. Levels of pro-inflammatory eicosanoids and cytokines were measured in plasma. The results showed that mildronate treatment decreased hepatic carnitine concentration and mitochondrial FA β-oxidation and induced severe triglyceride accumulation accompanied by elevated systemic inflammation. Surprisingly, inclusion of EPA in the diet exacerbated the mildronate-induced triglyceride accumulation. This was accompanied by a considerable increase of EPA accumulation while decreased total n-3/n-6 ratio in liver. However, inclusion of EPA in the diet attenuated the mildronate-induced mRNA expression of inflammatory genes in adipose tissue. Taken together, dietary supplementation with EPA exacerbated the triglyceride accumulation induced by impaired mitochondrial FA β-oxidation. Thus, further thorough evaluation of the potential risk of EPA supplementation as a therapy for NAFLD associated with impaired mitochondrial FA oxidation is warranted.



## 1. Introduction

Non-alcoholic fatty liver disease (NAFLD), characterized by excessive accumulation of hepatic triglycerides (TG) in the absence of alcohol consumption, represents the most common chronic liver disease in the Western world [1]. Dysregulated mitochondrial metabolism is central in NAFLD, and it has generally been assumed that limited mitochondrial FA β-oxidation is a common feature in NAFLD [2,3]. By contrast, a recent study provided evidence that NAFLD may be linked to increased adipose lipolysis, *de novo* hepatic lipogenesis and saturated lipoprotein triglyceride export [4]. This study also revealed elevated *in vivo* hepatic mitochondrial oxidative metabolism in human subjects with chronic insulin-resistance related NAFLD [4]. Still impaired hepatic mitochondrial FA β-oxidation may in some situations precede insulin-resistance and NAFLD due to exposure to drug, toxicants and environmental contaminants thereby triggering subsequent TG accumulation in liver. Moreover, even in the state of chronic NAFLD associated with exacerbated adipose

*Abbreviations:* ACC, acetyl-coenzyme A carboxylases; ACO, acetyl-coenzyme A oxidase; ALT, alanine aminotransferase; APC, adipocyte; AST, aspartate aminotransferase; ATGL, adipose triglyceride lipase; CD68, cluster of differentiation 68; COX-2, cyclooxygenase-2; CPT1, carnitine palmitoyltransferase I; CYP1A1, cytochrome P450 1A1; DHA, docosahexaenoic acid; D6D, delta-6-desaturase; ELOVL, fatty acid elongase; EPA, eicosapentaenoic acid; eWAT, epididymal white adipose tissue; FAS, fatty acid synthase; FFA, free fatty acid; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GPR120, G protein-coupled receptor 120; GPX1, glutathione peroxidase; HF, high fat; HSL, hormone sensitive lipase; IL-6, interleukin-6; iWAT, inguinal white adipose tissue; LCITMS, liquid chromatography ion-trap mass spectrometry; LCPUFA, long-chain polyunsaturated fatty acid; LTB, leukotriene B; MAO, monoamine oxidase; MEPG-1, macrophage expressed gene 1; NAFLD, non-alcohol fatty liver disease; PFAOS, peroxisomal fatty acid oxidation system; PGE, prostaglandin E; PPAR, peroxisome proliferator-activated receptors; ROS, reactive oxygen species; SCD1, stearoyl-coenzyme A desaturase-1; SREBP, sterol regulatory element binding protein; TBARS, thiobarbituric acid reactive substances; TBP, TATAA-box binding protein; TG, triglyceride; TNF-α, tumor necrosis factor-α; UCP, uncoupling protein; VEGFR, vascular endothelial growth factor receptor

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lipolysis, impairment of hepatic mitochondrial FA β-oxidation might aggravate the clinical manifestations of NAFLD.

Low levels of omega-3 (n-3) long chain polyunsaturated fatty acids (LCPUFA) and high omega-6 (n-6)/n-3 PUFA ratio have been observed in the patients and animals with NAFLD [5]. A number of studies have indicated that intake of n-3 LCPUFA prevents hepatic accumulation of TG, partly through increasing hepatic FA β-oxidation and reducing inflammation in the progress of NAFLD [6–9]. Hence, dietary supplement of n-3 LCPUFA was recently recommended as a promising novel therapy for NAFLD [10,11].

Several studies have suggested that the effect of n-3 LCPUFA in reducing both triglyceride accumulation and secretion is primarily mediated by eicosapentaenoic acid (EPA) [8,12,13], and in fact, EPA is presently the only n-3 LCPUFA that is used clinically as a single-agent treatment for hypertriglyceridemia [8]. In keeping with earlier studies [9,14], our recent work has demonstrated that EPA, but not docosahexaenoic acid (DHA), is efficiently metabolized through the hepatic mitochondrial FA β-oxidation system [15,16]. Accordingly, decreased mitochondrial FA β-oxidizing capacity could increase EPA accumulation in liver and thereby competitively decrease the synthesis of pro-inflammatory factors such as arachidonic acid-derived prostaglandin $E_2$ ($PGE_2$) [16,17].

Thus, increased mitochondrial FA β-oxidation might be a prerequisite for EPA to prevent accumulation of triglycerides in the liver and thereby protect against steatosis and non-alcoholic steatohepatitis. In this setting, inhibition of mitochondrial FA β-oxidation might aggravate steatosis, but increase the relative amount of EPA in the liver. Therefore, it is of importance to examine whether dietary EPA supplementation attenuates or exacerbates the progress of NAFLD in conditions where FA β-oxidation is impaired.

Recently, a novel experimental NAFLD model was developed based on the use of mildronate, a cardioprotective drug used to facilitate glycolysis during ischemia by inhibiting carnitine synthesis and carnitine reabsorption in kidneys. Mildronate treatment led to accumulation of EPA [17]. In a follow-up experiment, after 10 days of mildronate treatment was found to reduce hepatic mitochondrial FA β-oxidizing capacity causing pronounced development of fatty liver upon fasting in normal low-fat diet fed Wistar rats [18]. Of note, in both studies, the rats were fed low-fat diets, whereas the effect of mildronate on EPA accumulation and NAFLD development in combination with a high-fat diet was not evaluated. Development of NAFLD is associated with intake of energy-dense high-fat containing foods [19], and altered food choice is an important factor associated with improved manifestations of NAFLD [20]. From a clinical point of view it would seem relevant to utilize a high-fat feeding regiment in the context of the experimental mildronate NAFLD model.

Accordingly, in the present study we performed a comprehensive investigation of the ability of mildronate to induce NAFLD in male C57BL/6J mice fed a high-fat diet with or without EPA. We examined the impacts of mildronate administration on the metabolism of EPA and the possible physiological consequences of EPA accumulation in the liver.

## 2. Materials and methods

### 2.1. Animals and treatment

Male C57BL/6J mice (6 weeks of age) were obtained from Taconic Europe (Ejby, Denmark). The animals were kept at a 12-h light/dark cycle in a temperature-controlled room at 22 °C. After acclimatization, individually housed animals were divided into 4 experimental groups ($n=9$) and fed a high-fat (HF) diet (35 wt.% fat, Supplemental Table 1) for 10 days. Two groups were fed the basal high fat (HF) diet whereas the two remaining groups were fed an EPA-supplemented HF diet (HF-EPA) where 3% stearic acid was replaced by EPA. One HF and HF-EPA group received daily 500 mg mildronate/kg body weight dissolved in 150 μl fructose solution (0.5%), and one HF and HF-EPA group received 150 μl fructose solution (vehicle). In a separate experiment, male C57BL/6J mice were divided into 4 groups ($n=8$) and fed a low-fat (LF) diet (7.5 wt.% fat, Supplemental Table 2) or a LF-EPA diet where 2.5% stearic acid was replaced by EPA. One LF and LF-EPA group received daily 500 mg mildronate/kg body weight and one LF and LF-EPA group received 150 μl fructose solution (vehicle). Administration of the solution was through voluntary oral consumption using cups provided daily. Animals were initially trained using the fructose solution for several days, so that during the experimental period the solutions were consumed within 15 min. Feed intake and body weight were recorded at 10:00 AM every second day throughout the experiments. At the end of the experiment, blood was collected, heparin-plasma prepared, and tissue dissected out and weighed from animals in the fed state. The animal experiments were approved by the National State Board of Biological Experiments with Living Animals (FOTS ID2284).

### 2.2. Plasma measurements and histology

Lipids, metabolites, and enzyme activities in plasma were determined by commercially available enzymatic kits as follows: alanine aminotransferase (ALT) and aspartate aminotransferase (AST) (Dialab, Vienna, Austria); free FAs, glucose and total triglycerides (MaxMat, Montpellier, France). ELISA kits were used to measure insulin, leptin, interleukin-6 (IL-6) and tumor necrosis factor-α (TNF-α) (Invitrogen Co., CA). Twenty carbon-FA-derived eicosanoids, prostaglandin $E_2$ ($PGE_2$), $PGE_3$, leukotriene $B_4$ ($LTB_4$), and $LTB_5$ were measured by liquid chromatography ion-trap mass spectrometry (LC/ITMS) as described elsewhere [21]. The paraffin sectioning and H&E staining of liver and epididymal white adipose tissue (eWAT) were performed as described [22].

### 2.3. Hepatic enzyme activities and total carnitine assay

[1-$^{14}$C]palmitate oxidation capacity was determined using whole liver homogenates in the absence of exogenous L-carnitine, and in isolated mitochondria in the presence of 0.5 mM exogenous L-carnitine [23]. CPT I activity was measured with L-[*methyl*-$^{14}$C]carnitine (Amersham Biosciences), as previously described [23]. Monoamine oxidase (MAO) was measured in liver homogenates and/or isolated mitochondria as indicated [24]. Total mitochondrial protein (mg/g liver) was calculated by dividing the activity of MAO expressed per g of liver (using homogenates) by the activity of corresponding enzymes expressed per mg of protein of isolated mitochondrial fractions. Carnitine-independent [1-$^{14}$C]palmitate oxidation and potassium cyanide (KCN)-insensitive palmitoyl-CoA-dependent NAD reduction reflecting the peroxisomal FA-oxidation, were directly measured in whole liver homogenates[23]. Total carnitine in liver was measured by a commercial kit (Biosentec, Toulose, France).

### 2.4. Lipid classes and FA composition

Hepatic lipids were extracted, separated and analyzed by gas chromatography (GC) and TLC as described previously [16,25]. The results of fatty composition were expressed as absolute concentration (mg/g liver), and relative abundance (% of total FAs) respectively.

### 2.5. mRNA expression

mRNA expression was analyzed by real-time quantitative PCR as described earlier [20]. The primer sequences are shown in Supplemental Table 2.

### 2.6. Statistics

The data presented are means ± SEM ($n=4$–9). Significant differences ($P<0.05$) of each variable were first determined using the