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High-protein diets prevent steatosis and induce hepatic accumulation of monomethyl branched-chain fatty acids☆,☆☆,★,★★

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Abstract

The hallmark of nonalcoholic fatty liver disease is steatosis of unknown etiology. To test how dietary protein decreases steatosis, we fed female C57BL/6 J mice low-fat (8 en%) or high-fat (42 en%) combined with low-protein (11 en%), high-protein (HP; 35 en%) or extra-high-protein (HPX; 58 en%) diets for 3 weeks. The 35 en% protein diets reduced hepatic triglyceride, free fatty acid, cholesterol and phospholipid contents to ~50% of that in 11 en% protein diets. Every additional 10 en% protein reduced hepatic fat content ~1.5 g%. HP diets had no effect on lipogenic or fatty acid-oxidizing genes except *Ppargc1α* (+30%), increased hepatic PCK1 content 3- to 5-fold, left plasma glucose and hepatic glycogen concentration unchanged, and decreased inflammation and cell stress (decreased *Fgf21* and increased *Gsta* expression). The HP-mediated decrease in steatosis correlated inversely with plasma branched-chain amino-acid (BCAA) concentrations and hepatic content of BCAA-derived monomethyl branched-chain fatty acids (mmBCFAs) 14-methylpentadecanoic (14-MPDA; valine-derived) and, to a lesser extent, 14-methylhexadecanoic acid (isoleucine-derived). Liver lipid content was 1.6- to 1.8-fold higher in females than in males, but the anti-steatotic effect of HP diets was equally strong. The strong up-regulation of PCK1 and literature data showing an increase in phosphoenolpyruvate and a decline in tricarboxylic acid cycle intermediates in liver reveal that an increased efflux of these intermediates from mitochondria represents an important effect of an HP diet. The HP diet-induced increase in 14-MPDA and the dietary response in gene expression were more pronounced in females than males. Our findings are compatible with a facilitating role of valine-derived mmBCFAs in the antisteatotic effect of HP diets.

Keywords: High-protein diet; Fatty liver; Fatty acid composition; Branched-chain keto acids; Branched-chain amino acids

1. Introduction

Nonalcoholic fatty-liver disease (NAFLD) is the most common chronic liver disease in affluent societies and the hepatic manifestation of the metabolic syndrome [1]. The hallmark of NAFLD is lipid accumulation in hepatocytes (steatosis) with a thus-far unknown etiology. Steatotic livers have an up to 2-fold increased rate of lipolysis, anaplerotic flux via pyruvate carboxylase, oxidative flux through the tricarboxylic acid (TCA) cycle, ketogenesis and gluconeogenesis [2,3], implying that a high lipid content of a liver or muscle cell increases fatty acid oxidation ("supply-side" metabolic control). Furthermore, incompletely oxidized lipotoxic by-products of fatty acid metabolism, such as free fatty acids (FFAs), (lyso-)phosphatidic acid, lysophospatidylcholines, ceramides and diacylglycerols, may accumulate in steatotic hepatocytes [4]. The subsequent loss of insulin sensitivity then allows an increased rate of gluconeogenesis and mitochondrial TCA cycle metabolism [5]. In agreement, steatosis is more strongly associated with key parameters of the metabolic syndrome, such as increased plasma insulin and very low-density lipoprotein (VLDL)

Abbreviations: BHB, β-hydroxybutyrate; en%, energy percent; g%, gram percent; HF, high fat (42 en%); HP, high protein (35 en%); HPX, high protein (58 en%); LF, low fat (8 en%); LP, low protein (11 en%); mmBCFA, monomethyl branched-chain fatty acid; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; PL, phospholipids (choline-esters); TC, total cholesterol; SFA, saturated fatty acid; MUFA, monounsaturated fatty acid; PUFA, polyunsaturated fatty acid.

☆ The authors declare that they have no competing interests.

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★ Supplemental Figs. 1–4 and Supplemental Tables 1–6 are available online.

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secretion, and decreased plasma adiponectin levels than with body mass index or visceral adipose tissue mass [6]. Moreover, fatty hepatocytes secrete mediators that activate the profibrotic stellate cells [7]. Finally, steatosis is a risk factor for patients undergoing major liver surgery [8]. For all these reasons, steatosis-reducing therapies should benefit patients with NAFLD.

Recently, we showed that a high-protein (HP) diet very efficiently reduces a high-fat (HF) diet-induced hepatic steatosis in male mice [9]. It was also shown that an HP diet decreased hepatic steatosis in obese female patients by 20% within just 4 weeks [10]. One of the prominent features in male mice fed an HP diet was the increased plasma concentration of branched-chain amino acids (BCAA) and the strong inverse correlation between plasma BCAA and hepatic triglyceride (TG) concentrations [9]. This selective increase in plasma BCAA concentration is well documented in rats on an HP diet [11–14]. BCAAs are transaminated to branched-chain keto-acids in many organs, but the liver plays a key role in their subsequent metabolism to branched-chain acyl-CoAs. Branched-chain acyl-CoAs are further catabolized in the TCA cycle or replace acetyl-CoA as primer for the synthesis of monomethyl branched-chain fatty acids (mmBCFAs). mmBCFAs are attractive candidates to bring about a lean liver, as they were already shown to be important modulators of lipid metabolism in *Caenorhabditis elegans* [15]. We, therefore, hypothesized that liver TG concentration correlated inversely with the accumulation of mmBCFAs in the liver. Female mice appear preferable over male mice to investigate this question, because the key enzyme branched-chain α-keto-acid dehydrogenase kinase is more highly expressed in female liver [16,17]. In this respect, it is also of interest that the HP diet that decreased hepatic steatosis in the human trial cited above [10] only included female patients. The aim of this study was, therefore, to determine if an HP diet was as effective in female as in male mice to reduce diet-induced steatosis and if BCAAs accumulate in the circulation and mmBCFAs in the liver of such mice.

2. Methods

Detailed protocols and details on materials used (Supplemental Table 1) are provided in Supplemental Materials.

2.1. Animals

Female C57BL/6J mice were purchased from Charles River (France) and housed in pairs. Twice weekly, food intake (difference between food offered and food left, corrected for spillage) and body weight were measured. After 3 weeks on the specified diet, mice were sacrificed in the morning (10:00 h–12:30 h) to avoid chronobiological effects. Mice had access to food and water until sacrifice. Mice were anesthetized by intraperitoneal injection of a solution containing (per kg body weight) 52.5 mg midazolam, 105 mg fluanisone, and 3.3 mg fentanyl-citrate, and subsequently exsanguinated by harvesting blood from the inferior caval vein. Liver and inguinal fat pads were sampled, weighed and snap-frozen for further analysis. The study was approved by the Committee for Animal Care and Use of Maastricht University.

2.2. Diets and experimental groups

Four semisynthetic diets (Supplemental Table 2) were purchased from Research Diets Inc (New Brunswick, NJ, USA). The diets were designed to test the effect of a low or a high dietary protein content (LP and HP, respectively) in a low-fat (LF) or HF diet. The LF/LP diet contained 8 en% fat and 11 en% protein; the LF/HP diet, 8 en% fat and 35 en% protein; the HF/LP diet, 42 en% fat and 11 en% protein; and the HF/HP diet, 42 en% fat and 35 en% protein. The 11 en% protein that is present in the LP diets meets the daily protein requirement of adult mice [18]. The LF/LP diet was the reference diet. In addition, a limited number of parameters were determined in male and female mice fed a carbohydrate-free HF (cocoa butter) diet (Research Diets Inc, New Brunswick, NJ, USA) that contained 58 en% protein (HF/HPX; Supplemental Table 2). Male C57Bl/6 J mice fed this diet had an average hepatic TG content of 0.22 mmol/g protein or 3.75 g%, which compares well with male mice fed a standard LF (8–12 en%), medium-protein (20–24 en%) diet (compiled data of previously published articles; Supplemental Fig. 1A).

2.3. Messenger RNA quantitation

Messenger RNA (mRNA) was quantified with real-time polymerase chain reaction (PCR) using 18S rRNA as reference. For graphic presentation, 18S-corrected values were divided by the mean of the male LF/LP group (=100%; [9]). Primer sequences and amplicon concentrations for each gene are given in Supplemental Tables 3 and 4, respectively.

2.4. Plasma analysis

At sacrifice, heparinized blood was obtained from the inferior caval vein and stored at −80°C. Plasma lipids, hormones, amino acids, ketone bodies and glucose were determined using commercially available kits (Supplemental Table 1) or established methods (see Online Supplemental Materials). Two days before sacrifice, whole-blood venous glucose was measured in 4-h fasted mice. Plasma amino acids were measured in 50 μl plasma as described [19].

2.5. Liver analysis

Lipid [TGs, FFAs, phospholipids (PLs) and total cholesterol (TC)] and protein concentrations in liver homogenates were determined using commercially available kits. The hepatic concentration of specific proteins was determined by Western blotting. Liver glycogen was determined as described [20].

2.6. Hepatic fatty acid composition

Liver tissue was homogenized using the IKA T10 UltraTurrax (Staufen, Germany). After sonication (twice at 8-W output, 40 J, on ice), protein concentration was measured and all samples were diluted to 2 mg/ml. Fatty acids from a 100-μg sample were directly transesterified to methylesters and analyzed by gas chromatography with flame ionization detection [21].

2.7. Statistical analysis

A two-way analysis of variance (ANOVA) was used to test for differences between the main effects of the dietary components that were varied (HP or HF) and their interactions (HFxHP). For pairwise comparisons between groups (LF/LP, LF/HP, HF/LP, HF/HP), a one-way ANOVA including a post hoc analysis was used. For data with equal variance, Tukey's test was used, whereas for the remaining cases, Games–Howell's test was applied. P values <.05 were considered significant and <.1 as indicating a trend. Error bars represent standard errors of the mean (S.E.M.).

3. Results

3.1. Energy intake and biometric response

Mice on an HF/LP diet consumed ~20% more food than mice on an HF/HP or an LF diet (P<.001; Table 1). Although weight gain and feed efficiency were highest on the LF/LP diet, the difference with the other diets did not reach significance due to the high variation. Mice on the LF diets had a slightly higher relative liver weight than those on the HF diets (P=.011), which is probably due to the higher glycogen