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Research paper

Hepatosteatosis in peroxisome deficient liver despite increased β-oxidation capacity and impaired lipogenesis

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ABSTRACT

Peroxisome deficiency in liver causes hepatosteatosis both in patients and in mice. Here, we studied the mechanisms that contribute to this lipid accumulation and to activation of peroxisome proliferator activated receptor α (PPARα) by using liver-specific *Pex5*$^{-/-}$ mice (*L-Pex5*$^{-/-}$ mice). Surprisingly, steatosis was accompanied both by increased mitochondrial β-oxidation capacity, confirming previous observations, and by impaired *de novo* lipid synthesis mediated by reduced expression of sterol regulatory element binding protein 1c and its targets. As a consequence, when challenged with a high fat diet, *L-Pex5*$^{-/-}$ mice were protected from adiposity. Hepatic fatty acid uptake was strongly increased whereas the expression of apolipoproteins and the lipoprotein assembly factor microsomal triglyceride transfer protein were markedly reduced resulting in reduced secretion of very low density lipoproteins. Most of these changes seemed to be orchestrated by the endogenous activation of PPARα, challenging the assumption that PPARα activation in hepatocytes requires fatty acid synthase dependent *de novo* fatty acid synthesis. Expression of cholesterol synthesizing enzymes and cholesterol levels were not affected in peroxisome deficient liver. In conclusion, increased fatty acid uptake driven by endogenous PPARα activation and reduced fatty acid secretion cause hepatosteatosis in peroxisome deficient livers.

1. Introduction

Hepatic peroxisomes play an essential role in intermediary lipid metabolism. Under normal conditions, their contribution in the degradation of the common C16–C18 fatty acids as compared to the role of mitochondria is limited (5–10% [1]; 26% [2] in rat liver). However, when the liver is overloaded with fatty acids such as in fasting conditions, ω-oxidation in the ER occurs, leading to the formation of dicarboxylic fatty acids which are subsequently β-oxidized in peroxisomes [3–6]. Furthermore, hepatic peroxisomes are of particular importance for the catabolism of branched chain fatty acids by α- and β-oxidation [6]. Peroxisomal β-oxidation is also necessary for the synthesis of C22 polyunsaturated fatty acids, for the degradation of very long chain fatty acids and the shortening of the side chain of cholesterol as part of the conversion to bile acids [6]. Also the subsequent conjugation of bile acids was shown to take place in peroxisomes [7–10]. Conflicting data have been published with regard to the presence of enzymes involved in cholesterol and isoprenoid synthesis in the peroxisomal compartment [8,11–13]. Cholesterogenesis was not impaired in liver and brain of neonatal *Pex5* knockout mice, a model with generalized peroxisome deficiency [14]. In contrast, in an analogous *Pex2* knockout model, it was reported that the expression of cholesterol synthesizing enzymes was increased and cholesterol levels were decreased in livers of juvenile mice [15,16].

To circumvent postnatal lethality of generalized *Pex5* knockout mice, mice with hepatocyte selective deletion of functional peroxisomes were generated [17]. These mice are viable into adulthood, but show mild growth retardation and develop a broad array of hepatic abnormalities. The livers of these mice are enlarged, have a fatty appearance and display microvesicular steatosis [17]. As expected, the livers contain an increased ratio of

Abbreviations: ACC, acetyl-CoA carboxylase; ACLY, ATP citrate lyase; Acox1, acyl-CoA oxidase; Adfp, adipose differentiation related protein; Apo, apolipoprotein; Cpt, carnitine palmitoyltransferase; DHA, docosahexaenoic acid; FASN, fatty acid synthase; FGF21, fibroblast growth factor 21; *L-Pex5*$^{-/-}$ mice, liver-specific *Pex5*$^{-/-}$ mice; MFP, multifunctional protein; NAFLD, nonalcoholic fatty liver disease; NASH, nonalcoholic steatohepatitis; Pex, peroxin; PPARα, peroxisome proliferator activated receptor α; SREBP1c, sterol regulatory element binding protein; SCAP, SREBP cleavage-activating protein; VLDL, very low density lipoproteins.

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immature C27 to mature C24 bile acids [17]. When administered a normal diet that contains low amounts of branched chain fatty acids or their precursors, the hepatic levels of phytanic and pristanic acid were slightly increased [17]. Although ether phospholipids, including plasmalogens, depend on peroxisomes for their synthesis, plasmalogen levels were not decreased in peroxisome deficient livers [17]. This is likely due to the ability of other cell types/tissues to synthesize the ether bond.

An interesting observation was the up regulation of MFP-1, CYP4A1 and LCAD [17] which are respectively involved in peroxisomal, ER and mitochondrial oxidation of fatty acids. Because they are all PPARα target genes, this suggested that endogenous ligands of this nuclear receptor accumulate in peroxisome deficient hepatocytes. We showed that this was accompanied by increased mitochondrial β-oxidation [3], which is in apparent contradiction with the lipid accumulation in peroxisome deficient hepatocytes. Although various fatty acids have previously been pinpointed as endogenous PPARα ligands [18,19], it was recently reported that a particular phospholipid, 1-C16:0-2-C18:1-phosphatidylcholine, may be the true activator [20]. Furthermore, it was proposed that in order to activate PPARα, new fatty acid synthesis in liver is necessary whereas the pool of 'old' fatty acids derived from adipose tissue is not capable to trigger transcriptional activity [21].

In the present paper we investigated the mechanisms that lead to steatosis in peroxisome deficient hepatocytes. We considered as well altered β-oxidation, *de novo* lipid synthesis, uptake of fatty acids and secretion of lipoproteins. Furthermore, we investigated the relationship between PPARα activation in peroxisome deficient livers and lipogenesis and cholesterogenesis.

2. Experimental procedures

2.1. Mouse breeding

The generation and characterization of *L-Pex5*$^{-/-}$ mice has been described elsewhere [17]. Littermates not expressing Cre-recombinase were used as control animals. Mice were bred in the conventional animal housing facility of the University of Leuven. Animals were maintained on a 12h light/12h dark schedule and were fed a standard rodent chow and water *ad libitum*. Unless stated otherwise, all mice were sacrificed in fed condition between 8 and 11 am, at an age between 10 and 15 weeks. Some mice were fed a high fat diet (Harlan Adjusted Kcal Diet (42% from fat), Harlan Teklad, Madison, US) for 6 weeks. All animal experiments were approved by the Institutional Animal Ethical Committee of the University of Leuven.

2.2. In vivo experiments

2.2.1. Whole body dual-energy X-ray absorptiometry (DEXA)

Body composition was analyzed *in vivo* by DEXA (PIXImus™ densitometer, Lunar Corp, Madison, WI, USA), using ultra-high resolution (0.18 × 0.18 pixels, resolution of 1.6 line pairs/mm) and software version 1.45.

2.2.2. VLDL-secretion

Mice were injected intravenously with 600 mg Tyloxapol (Sigma, Belgium) per kg body weight. Blood was taken via retro-orbital puncture before injection and 90 and 180 min after injection. Triglyceride levels in plasma were enzymatically quantified as previously described [22].

2.2.3. Utilization of glucose in lipid synthesis

Overnight fasted mice were intravenously injected with 0.33 μCi deoxy-D-glucose 2-[1-^{3}H] and 1.5 mg glucose per g body weight. 40 min after injection, liver tissue was collected and rinsed with ice-cold 1 mM EDTA/PBS. After Bligh-Dyer extraction [23], the ^{3}H-glucose derived label that was incorporated in lipids was quantified by liquid scintillation counting.

2.3. Protein analysis: western blotting

Western blotting experiments were conducted as described previously [24]. HRP-labeled secondary antibodies were used and detection was performed using ECL plus detection kit (Amersham). Primary antibodies were purchased at Novus Biologicals (Littleton, USA) (CD36) and Cell Signaling Technology (ACC and phosphorylated ACC).

2.4. Lipid/metabolic analysis

2.4.1. Liver and plasma lipid metabolites

Plasma and liver free fatty acid concentrations were measured with a NEFA-C kit (Wako Diagnostics, Richmond, USA) according to the manufacturer's protocol. Triglycerides were measured in a Bligh-Dyer extract of liver and in plasma as described above (2.2.1). Liver cholesterol and cholesterylesters were measured using a colorimetric assay as described previously [25]. Plasma FGF21 levels were quantified using a commercial RIA-kit (Phoenix Europe GMBH).

2.4.2. Phosphatidylcholine profiling

Phospholipids were analyzed by electrospray ionization tandem mass spectrometry as previously described [26]. Data were expressed as percentage of total phosphatidylcholine (PC) content.

2.5. Fatty acid uptake and synthesis by hepatocyte cultures

Hepatocytes were isolated as previously described [3]. To visualize uptake of fatty acids, hepatocytes were incubated one day after isolation for 3 h in serum-free medium, washed with PBS and incubated with 100 nM BODIPY-C_{16} (Invitrogen) for 3 min. Subsequently, cells were washed with PBS, fixed with paraformaldehyde and analyzed with fluorescence microscopy.

Hepatocytes were incubated with [2-^{14}C] acetate (57 mCi/mmol; 1 μCi/dish; Amersham International) during 4 h and lipids were extracted by the Bligh-Dyer method. The incorporation of ^{14}C-acetate was measured by liquid scintillation counting. For data normalization, DNA content was determined in cells collected in homogenization buffer (0.05 M Na_2HPO_4/NaH_2PO_4 buffer, 2.0 M NaCl, 2 mM EDTA, pH 7.4) by measuring fluorescence after incubation with Hoechst 33258 (1.6 μM) using a Fluostar fluorimeter (excitation at 355 nm, emission at 460 nm).

2.6. RNA analysis

2.6.1. RNA isolation and quantitative real-time RT-PCR

Total RNA was isolated from liver using the Trizol® reagent (Invitrogen, Merelbeke, Belgium). All subsequent steps were performed in accordance with the manufacturer's specifications. Three microgram of RNA was used for generation of cDNA using superscript reverse transcriptase (Invitrogen, Merelbeke, Belgium) and 0.5 μg oligo-dT12-18, followed by real-time PCR using an ABI PRISM 7500 PCR instrument (Applied Biosystems, Lennik, Belgium). Primers and probes were designed using Primer Express Software (Applied Biosystems, Lennik, Belgium) (Adfp forward primer: CCACTGTGTTGAGCACATCGA, Adfp reverse primer: GTCTGGAGCTG CTGGGTCA, Adfp probe: ACGTACTCTGGCTATCGCCCGCAA; CD36 forward primer: GCCAAGCTATTGCGACATGA, CD36 reverse primer: TCTCAATGTCCGAGACTTTTCAAC, CD36 probe: ACAGACGCAGCCTC