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Quantitation of the rates of hepatic and intestinal cholesterol synthesis in lysosomal acid lipase-deficient mice before and during treatment with ezetimibe

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ABSTRACT

Esterified cholesterol (EC) and triglycerides, contained within lipoproteins taken up by cells, are hydrolysed by lysosomal acid lipase (LAL) in the late endosomal/lysosomal (E/L) compartment. The resulting unesterified cholesterol (UC) is transported via Niemann-Pick type C2 and C1 into the cytosolic compartment where it enters a putative pool of metabolically active cholesterol that is utilized in accordance with cellular needs. Loss-of-function mutations in LIPA, the gene encoding LAL, result in dramatic increases in tissue concentrations of EC, a hallmark feature of Wolman disease and cholesteryl ester storage disease (CESD). The lysosomal sequestration of EC causes cells to respond to a perceived deficit of sterol by increasing their rate of cholesterol synthesis, particularly in the liver. A similar compensatory response occurs with treatments that disrupt the enterohepatic movement of cholesterol or bile acids. Here we measured rates of cholesterol synthesis in vivo in the liver and small intestine of a mouse model for CESD given the cholesterol absorption inhibitor ezetimibe from weaning until early adulthood. Consistent with previous findings, this treatment significantly reduced the amount of EC sequestered in the liver (from 132.43 ± 7.35 to 70.07 ± 6.04 mg/organ) and small intestine (from 2.78 ± 0.21 to 1.34 ± 0.09 mg/organ) in the LAL-deficient mice even though their rates of hepatic and intestinal cholesterol synthesis were either comparable to, or exceeded those in matching untreated *Lal*$^{-/-}$ mice. These data reveal the role of intestinal cholesterol absorption in driving the expansion of tissue EC content and disease progression in LAL deficiency.

1. Introduction

There is a vast literature defining the role that the liver and small intestine play in the regulation of whole-body cholesterol metabolism and the plasma lipoprotein composition [1–6]. Like most other organs, the liver and small intestine actively synthesize cholesterol although at rates that vary with multiple factors. This is especially true in the liver where the synthesis rate can fluctuate over an extraordinary range in response to shifts in dietary cholesterol intake or treatment with different classes of cholesterol-lowering agents, particularly statins, bile acid sequestrants and cholesterol absorption inhibitors [7–12]. Changes in cholesterol synthesis rates can also occur because of mutations in genes that encode enzymes at specific steps in the biosynthetic pathway [13], or that regulate the absorption, transport, degradation, storage and excretion of cholesterol [14–18]. Mutations in three particular genes, LIPA, NPC1 and NPC2, which together regulate cholesterol trafficking through the late endosomal/lysosomal compartment of every cell, have far reaching consequences in most organ systems. In the case of NPC2, and especially NPC1, mutations lead to multisystem disease including neurodegeneration, liver failure and pulmonary dysfunction [19]. Mutations in LIPA, the gene that encodes LAL, result

Abbreviations: ALT, alanine aminotransferase; bw, body weight; CESD, cholesteryl ester storage disease; EC, esterified cholesterol; E/L, endosomal/lysosomal; ERT, enzyme replacement therapy; Fas, fatty acid synthase; Hmgcr, hydroxymethylglutaryl coenzyme A reductase; Hmgcs, hydroxymethylglutaryl coenzyme A synthase; LAL, lysosomal acid lipase; Lipa, gene that encodes LAL; Ldlr, low-density lipoprotein receptor; Npc1l1, Niemann-Pick C1-Like 1; Srebp2, sterol regulatory element-binding protein 2; UC, unesterified cholesterol; Soat, sterol *O*-acyltransferase; VLDL, very low density lipoprotein.

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in either Wolman disease (WD), or cholesteryl ester storage disease (CESD). Whereas WD is a severe, early onset illness caused by complete loss of LAL activity, CESD is a milder, later-onset disease resulting from partial LAL deficiency [20–22].

In NPC1 or NPC2 deficiency, unesterified cholesterol (UC) becomes sequestered in the late endosomal/lysosomal compartment thus preventing its movement elsewhere in the cell for further utilization. Such entrapment of UC causes a perceived shortage of cholesterol in cells leading to a compensatory increase in cholesterol synthesis [14]. The same scenario is seen in LAL deficiency except that in this disorder it is esterified cholesterol (EC) that is sequestered in the E/L compartment [20–23]. Our studies in a mouse model for CESD showed a profound compensatory increase in hepatic cholesterol synthesis [23]. The greatly elevated rate per gram of liver, combined with the pronounced increase in liver mass, were largely responsible for driving the elevation in whole-body cholesterol synthesis in this model even though increased synthesis in other organs, including the small intestine, contributed to this elevation.

Although an enzyme replacement therapy (ERT), Sebelipase alfa, is now available for treating LAL deficiency [24,25], various classes of cholesterol-lowering agents have proven useful for managing the dyslipidemia often seen in CESD patients [26–28]. One of these is ezetimibe, a potent and selective inhibitor of intestinal sterol absorption that is widely used in combination with statins, or as a monotherapy, for dyslipidemia management in the general population [29,30]. A more recent study demonstrated efficacy of ezetimibe monotherapy in children with heterozygous familial or nonfamilial hypercholesterolemia [31]. Ezetimibe is now an established therapy for sitosterolemia, a rare sterol storage disease [32]. It has also been shown to reduce biliary cholesterol saturation in humans and animal models [7,8,33]. Studies from multiple labs using various types of animal models for non-alcoholic fatty liver disease (NAFLD) implied a therapeutic benefit of ezetimibe in this disorder [34–36]. However, a recent clinical trial in patients with non-alcoholic steatohepatitis (NASH) given ezetimibe did not find a reduction in liver fat content [37]. Although the mouse model for CESD that we acquired does not manifest elevated plasma total cholesterol levels, we used it for an exploratory study to determine whether the imposition of a chronic block of intestinal cholesterol absorption by ezetimibe from the time of weaning had any impact on liver mass and EC content by the time the mutants reached early adulthood [38]. The treated mutants showed a reduction in both their degree of hepatomegaly and the level of EC sequestration in the liver, along with a clear fall in plasma ALT activity. These decisive effects raised several questions, particularly as blocking cholesterol absorption with ezetimibe is known to lead to a dramatic compensatory increase in hepatic, intestinal and whole-body cholesterol synthesis in other types of animal models [7,8] and humans [39].

The principal objective of these studies then was to use an established in vivo technique to determine what happens to the already elevated rates of cholesterol synthesis in the liver and small intestine in LAL-deficient mice when they have a sustained pharmacological block of their cholesterol absorption pathway. This was part of a broader goal of better understanding the interrelationship between intestinal and hepatic cholesterol metabolism in CESD, and of further exploring the potential use of ezetimibe as an adjunctive therapy for this rare disorder.

2. Materials and methods

2.1. Animals and diets

Lal$^{+/+}$ and *Lal*$^{-/-}$ mice were generated from heterozygous breeding stock, all on an FVB/N strain background. The litters were weaned at 21 days and genotyped before that age using an ear notch. Except for an initial experiment using different doses of ezetimibe, all studies were carried out in females only. The ground form of a cereal-based, low-cholesterol rodent chow diet (No. 7001, Envigo:Teklad, Madison, WI) was used in all experiments. This formulation had an inherent cholesterol content of 0.02% (w/w) and a crude fat content of not less than 4% [38]. In our previously published experiments with ezetimibe treated Lal-deficient mice, only males were studied, and the dose of ezetimibe was set at ~20 mg/day/kg bw. This dose was determined on the basis of a food intake of approximately 160 g/day/kg bw that was measured in another project using young adult *Ldlr*$^{-/-}$ mice and their wildtype controls given the chow diet alone [7]. For the current studies we first investigated the impact of varying the dose of ezetimibe on liver mass and cholesterol content in Lal-deficient mice. As shown in Table 1, a modest but significant reduction in the degree of hepatomegaly and hepatic total cholesterol concentration was seen at a dose of 5 mg/day/kg bw. A dose of 20 mg/day/kg bw was considerably more efficacious. Doubling this dose resulted in about the same degree of reduction in hepatic cholesterol content. Therefore, it was determined that 20 mg/day/kg bw would be used in the subsequent studies. In all experiments, the mice were housed in groups of 3 or 4 per cage, their food intake and stool output were monitored daily, and their body weights were measured at weekly intervals. The period of ezetimibe treatment was 28–30 days starting on the day of weaning. Hence, their age at the time of study was 49–51 days. The mice were provided their respective diets ad libitum and studied in the fed state towards the end of the dark phase of the lighting cycle. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

2.2. Measurement of rate of cholesterol and fatty acid synthesis in the liver and small intestine, and of cholesterol synthesis in the whole animal

These rates were measured in vivo using [^{3}H]water as detailed elsewhere [15,40]. Custom generated [^{3}H]water at a concentration of 5 Ci/ml (PerkinElmer Life Sciences) was subsequently diluted in sterile sodium chloride solution (0.9% w/v) to ~200 mCi/ml. One h after the mice were administered ~40 mCi of [3H] water (~0.2 ml) intraperitoneally, the liver and whole small intestine were removed, rinsed, blotted, and weighed. They were then saponified and the labeled sterols extracted and quantitated as described [40]. The rate of cholesterol synthesis in each organ was calculated as nmol of water incorporated into sterols/h/g wet weight of tissue (nmol/h/g). In one study, the residual carcass was digested and its labeled sterols isolated. The combined [^{3}H]sterol contents of the liver, small intestine and carcass yielded a measure of whole-animal sterol synthesis (nmol/h). The rate of hepatic and intestinal fatty acid synthesis was determined with the same tissue extracts used for isolation of the tritiated sterols [15]. These rates were expressed in the same way as those for cholesterol synthesis in these organs (nmol/h/g).

2.3. Quantitation of total, unesterified and esterified cholesterol in tissue and plasma, and of plasma ALT activity

After exsanguination, the liver and entire small intestine were removed, rinsed, blotted and weighed. Depending on the planned measurements, aliquots of liver, and the whole small intestine were placed in chloroform:methanol (2:1 v/v) for determination of the tissue concentrations (mg/g) of esterified (EC) and unesterified cholesterol (UC) using a combination of column and gas chromatography as described [41]. When only