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Impact of loss of SOAT2 function on disease progression in the lysosomal acid lipase-deficient mouse

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ABSTRACT

Although only a small proportion of cholesterol in the body is esterified, in several diseases marked expansion of the esterified cholesterol (EC) pool occurs. These include Wolman disease (WD) and Cholesteryl Ester Storage Disease (CESD) which both result from mutations in LIPA, the gene that encodes lysosomal acid lipase (LAL). The respective contributions that our three cholesterol esterifying enzymes make to EC production, especially in disorders like CESD, are not well defined. The current studies represent a detailed exploration of our earlier findings in young male LAL-deficient mice also missing sterol O-acyltransferase 2 (SOAT2, also called ACAT2). Here we show that, even as they aged, male and female *Lal*$^{-/-}$: *Soat2*$^{-/-}$ mice, compared to *Lal*$^{-/-}$: *Soat2*$^{+/+}$ littermates, had appreciably less hepatomegaly as well as a marked reduction in the level of sequestration of EC, in liver transaminase activities, and in hepatic mRNA expression levels for markers of inflammation. Loss of SOAT2 function also dramatically curtailed EC entrapment in the small intestine of the LAL-deficient mice. Together, these data imply that SOAT2 inhibition, if applied concurrently with enzyme replacement therapy for LAL deficiency, may blunt the re-esterification of newly released unesterified cholesterol thereby improving clinical outcomes.

1. Introduction

Over a broad range of mammalian species, including the mouse and human, the total cholesterol concentration across all organs averages about 2 mg per gram wet weight of tissue [1,2]. The proportion of cholesterol throughout the body that is esterified is quite small even though in the plasma and organs like the adrenal glands the bulk of the cholesterol is esterified [3,4]. Most of the cholesterol in exogenous sources is unesterified [5]. Even if this were not the case, little, if any, of the EC present in tissues originates from dietary intake because pancreatic cholesterol esterase hydrolyzes the EC present in foodstuffs [5]. Thus, the EC distributed throughout the body is generated from three esterifying enzymes, one of which is sterol *O*-acyltransferase 1 (SOAT1) present in steroidogenic tissues, kidneys, sebaceous glands and macrophages [6,7]. Another enzyme is SOAT2 (sterol O-acyltransferase 2, or ACAT2) which is expressed in hepatocytes and enterocytes [8–10]. The role of SOAT2 in generating EC that is incorporated directly into atherogenic lipoproteins has made it an attractive target in dyslipidemia management and prevention [11]. In the plasma compartment, cholesterol esterification is facilitated by lecithin cholesterol acyltransferase (LCAT) [12]. Loss of LCAT function results in a precipitous fall in circulating cholesterol levels, particularly in high density lipoproteins (HDL) [13,14].

Tissue EC levels are elevated in a number of rare diseases [3] including Tangier disease [15], Wolman disease (WD) and Cholesteryl Ester Storage Disease (CESD) [16,17]. These latter disorders result from mutations in LIPA, the gene that encodes lysosomal acid lipase (LAL) [18]. This enzyme hydrolyzes EC and triacylglycerol contained in various types of lipoproteins cleared from the circulation by receptor-mediated and bulk-phase endocytosis [19,20]. WD is a severe, early-onset illness caused by complete loss of LAL activity and typically leads to death in infancy, whereas CESD is a milder, later onset disease in which some residual LAL activity remains. A spontaneous rat model for WD [21], and a genetically engineered mouse model for CESD, have been invaluable in characterizing the biochemical, histological and molecular changes resulting from a disruption of LAL function [22,23], and in evaluating an enzyme replacement therapy for LAL deficiency [24,25]. Sebelipase alfa is now approved for the treatment of adult and pediatric patients with this rare disorder [26,27].

The exceptionally high level of sequestration of EC in the liver in

Abbreviations: CESD, cholesteryl ester storage disease; DKO, double knockout; EC, esterified cholesterol; ERT, enzyme replacement therapy; LAL, lysosomal acid lipase; LCAT, lecithin:cholesterol transferase; SOAT1, sterol *O*-acyltransferase 1; SOAT2, sterol O-acyltransferase 2; UC, unesterified cholesterol

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LAL deficiency reflects the fact that this organ is not only the major site of clearance of LDL and very low density lipoprotein remnants (VLDLr), all of which contain appreciable amounts of EC, but it also receives essentially all of the cholesterol that is absorbed from the small intestine, 70–80% of which is esterified [28,29]. Therefore, one of our first attempts to manipulate hepatic EC sequestration rates in LAL-knockout mice was to give them ezetimibe, a potent intestinal sterol absorption inhibitor, in their diet from the time of weaning until early adulthood. This manipulation culminated in a surprising degree of contraction in whole liver cholesterol content that reflected the combined effects of a reduction in both liver mass and EC concentration [30]. These findings prompted further studies in which we explored how genetic deletion or pharmacological suppression of SOAT2 in LAL-deficient mice impacted the level of disease severity in early adulthood [31,32]. The remarkable benefit that accrued from both strategies underscored the need to now determine whether the benefits of SOAT2 suppression in LAL deficiency were gender independent and prevailed with age.

2. Materials and methods

2.1. Animals and diets

The breeding stock ($Lal^{+/-}$: $Soat2^{+/-}$) used to generate the mice for these studies were derived from crossing $Lal^{+/-}$: $Soat2^{+/+}$ (pure FVB-1 background) with $Lal^{+/+}$: $Soat2^{+/-}$ (C57BL/6: 129/Sv) mice. The LAL heterozygous breeding stock were kindly provided by Drs. Greg Grabowski and Hong Du at the Children's Hospital Research Foundation in Cincinnati. SOAT2 heterozygous mice were originally purchased from the Jackson Laboratory. Large numbers of offspring were generated from the $Lal^{+/-}$: $Soat2^{+/-}$ breeding stock, with approximately equal numbers of males and females of the four required genotypes ($Lal^{+/+}$: $Soat2^{+/+}$, $Lal^{+/+}$: $Soat2^{-/-}$, $Lal^{-/-}$: $Soat2^{+/+}$, and $Lal^{-/-}$: $Soat2^{-/-}$) ultimately becoming available for the various sets of planned measurements. Newly generated $Lal^{+/-}$: $Soat2^{+/-}$ progeny were retained to replenish the breeding colony. The genotypes of the mice were determined at or before weaning at 21 or 22 days. All mice were weaned onto a low-cholesterol, low-fat, cereal-based rodent chow diet which they received ad libitum [23]. They were group housed in a light-cycled room and were studied in the fed state towards the end of the dark phase of the lighting cycle. With the exception of one experiment involving milk collection from the stomachs of 7-day old pups, the age of the mice at study ranged from 22 to 120 days. This research was conducted in full conformity with the PHS Policy on Humane Care and Use of Laboratory Animals. All experiments were approved by the Institutional Animal Care and Use Committee at the University of Texas Southwestern Medical Center.

2.2. Quantitation of esterified and unesterified cholesterol in liver and small intestine, of total cholesterol in plasma and whole animal, and of triacylglycerol in liver

The mice were weighed, anesthetized, and exsanguinated from the lower vena cava into a heparinized syringe. Depending on their gender and age, the liver and small intestine were excised, rinsed, blotted on filter paper, and weighed. Aliquots of liver from multiple lobes and the entire small intestine were added directly to ~40 ml of chloroform: methanol (2:1 v/v) for later measurement of the concentrations of unesterified and esterified cholesterol, and, in the case of the liver, triacylglycerol as well. None of the tissue aliquots or whole intestines intended for the measurement of the esterified and unesterified fractions were placed in liquid nitrogen before being added to chloroform: methanol because this can result in an error in the ratio of esterified to unesterified cholesterol [33]. In the study involving 52-day old male mice, the residual carcass was weighed and added to ~150 ml of alcoholic KOH for subsequent measurement of total cholesterol content. Plasma from the 52-day old male mice was used for the determination of the total cholesterol concentration. For both the 52- and 100-day male old mice, and the 52-and 120- day old female mice, aliquots of chilled plasma were sent immediately to a commercial laboratory for measurement of ALT and AST activities. To determine the proportion of cholesterol in the milk consumed by suckling pups that was esterified, the solidified milk in the stomachs of five 7-day old mice from different litters was harvested, weighed, and placed in chloroform: methanol (2:1 v/v).

The methods for the extraction, separation and quantitation of the esterified and unesterified cholesterol fractions using column and gas chromatography have been described in detail elsewhere [34], as have the techniques for determining hepatic triacylglycerol content [35], the plasma cholesterol concentration and whole body cholesterol content [36]. Plasma lipoprotein composition was not determined. The amount of EC, UC or triacylglycerol in tissue was expressed as either mg/g wet weight of tissue, or/and mg per whole organ. Plasma total cholesterol concentrations were expressed as mg/dl, and plasma transaminase activities as units/L. Whole body cholesterol content was expressed as mg/100 g body weight. No tissues were taken for histology.

2.3. Relative mRNA expression analysis

In the study with the 52-day old male mice, an aliquot of liver, taken from the core of the largest lobe immediately after the organ had been weighed, was frozen in liquid nitrogen. mRNA levels were measured by qPCR using a Bio-Rad CFX384 Real-Time PCR Detection System. The primer sequences used to measure the mRNA levels for four genes that serve as markers of either macrophage presence (Soat1) (sterol *O*-acyltransferase 1), or inflammation Mip1α (chemokine (c-c motif) ligand 3), CD11c (integrin alpha x), and Tnfα (tumor necrosis factor alpha) are given in an earlier publication [23]. All analyses were determined by the comparative cycle number at threshold method [37]. The mRNA levels were normalized to cyclophilin, and the values for each mouse were then expressed relative to that obtained for the matching $Lal^{+/+}$: $Soat2^{+/+}$ controls, which, in each case, was arbitrarily set at 1.0.

2.4. Analysis of data

Values are the mean ± SEM for the specified number of animals. GraphPad Prism 6.02 software (GraphPad, San Diego) was used for all statistical analyses. Differences between mean values were tested for statistical significance ($p < .05$) by either a one-way analysis of variance (ANOVA) or a two-way ANOVA with genotype and age as variables and applying Tukey's multiple comparison test.

3. Results

3.1. Diminished levels of esterified cholesterol in livers of $Lal^{-/-}$: $Soat2^{-/-}$ mice were evident at the time of weaning

One objective of these new studies was to investigate whether the impressive benefit of SOAT2 deficiency seen in male LAL knockout mice in early adulthood [31] persisted with age. It was also important to compare liver mass and esterified cholesterol (EC) concentrations in the DKO mice and their $Lal^{-/-}$: $Soat2^{+/+}$ littermates at the time of weaning, given that during at least the first two weeks after birth, mouse pups consume only milk. Earlier studies showed that, at 10 days of age, their milk intake was 0.96 ml per day [38], and also that mouse milk had a total lipid content of 41.6% and a total cholesterol concentration of 36.9 mg/dl [39]. In milk harvested from the stomachs of 7-day old pups in the current studies, 25 ± 6% was esterified. Hence, in that period of rapid organ development, the intake of cholesterol and lipids by mice generally is far higher than is the case when they are weaned onto a conventional rodent chow diet. At 22 days of age, there was a trend ($p > 0.05$) toward less hepatomegaly in the $Lal^{-/-}$: $Soat2^{-/-}$

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