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Ezetimibe restores biliary cholesterol excretion in mice expressing Niemann–Pick C1-Like 1 only in liver

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ABSTRACT

Niemann-Pick C1-Like 1 (NPC1L1) is highly expressed in the small intestine across mammalian species and is the target of ezetimibe, a potent cholesterol absorption inhibitor. In humans, NPC1L1 is also expressed in the liver. We found that transgenic overexpression of NPC1L1 in the wild-type mouse liver inhibits biliary cholesterol secretion and raises blood cholesterol, which can be reversed by ezetimibe treatment. Unfortunately, the high expression of endogenous NPC1L1 in the intestine hampered a definitive establishment of the role of hepatic NPC1L1 in cholesterol metabolism and ezetimibe action in the liver because intestinal NPC1L1 dramatically influences cholesterol homeostasis and is a target of ezetimibe. To circumvent this obstacle, we crossed liver-specific NPC1L1 transgenic mice to NPC1L1 knockout (L1-KO) mice and created a mouse line expressing no endogenous NPC1L1, but human NPC1L1 in liver only ($L1^{LivOnly}$ mice). Compared to L1-KO mice, $L1^{LivOnly}$ mice on a 0.2% cholesterol diet showed significantly increased hepatic and plasma cholesterol, and despite a 90% reduction in biliary cholesterol excretion, their fecal cholesterol excretion remained completely unaltered. Remarkably, 4 days of ezetimibe treatment significantly restored biliary cholesterol secretion in $L1^{LivOnly}$ mice. These findings demonstrated a direct role of hepatic NPC1L1 in regulating biliary cholesterol excretion and hepatic/blood cholesterol levels, and unequivocally established hepatic NPC1L1 as a target of ezetimibe.

1. Introduction

Cholesterol is essential for the growth and function of all mammalian cells. However, elevated blood cholesterol causes atherosclerotic cardiovascular diseases, the No. 1 killer in developed countries [1]. The cholesterol level in a body is determined by the integrated responses to cholesterol fluctuations of three major metabolic pathways, including de novo biosynthesis, intestinal absorption, and biliary/fecal excretion [2]. The molecular mechanism underlying the regulation of cholesterol biosynthesis has been elucidated elegantly by Drs. Brown and Goldstein's group in the last decade of the 20th century [3], and the key molecules responsible for hepatobiliary cholesterol secretion and intestinal cholesterol absorption have been identified at the dawn of the 21st century [4–8]. It is now well understood that cholesterol biosynthesis is tightly regulated by the membrane-bound transcription factor sterol regulatory element-binding protein (SREBP)-2 [3]; hepatobiliary cholesterol secretion is controlled by the heterodimer of two ATP-binding cassette (ABC) half-transporters G5 and G8 (ABCG5/G8) at the canalicular membrane of hepatocytes [4–7]; and intestinal cholesterol absorption is mediated by Niemann-Pick C1-Like 1 (NPC1L1) [8].

NPC1L1 is a polytopic transmembrane protein. Five of its transmembrane domains compose a sterol-sensing domain (SSD), a region conserved in many proteins involved in cholesterol metabolism [8–10]. In 2000, NPC1L1 was identified as a homolog of Niemann-Pick C1 (NPC1) [9]. Mutations in NPC1 cause Niemann–Pick Disease Type C1, an autosomal recessive genetic disease that is characterized by accumulation of free cholesterol and other lipids in lysosomes [11,12]. This observation suggests that NPC1L1 may be implicated in cellular trafficking of cholesterol and other lipids. In 2004, Altmann and his colleagues identified NPC1L1 as the target of ezetimibe (commercially known as Zetia) [8], a potent intestinal cholesterol absorption inhibitor that has now been widely used to lower blood cholesterol [13–17]. They showed that NPC1L1 is highly expressed in the small intestine, and that NPC1L1 knockout (L1-KO) mice display a substantial reduction in intestinal cholesterol absorption, a degree similar to that seen in ezetimibe-treated mice [8]. Subsequent protein–drug binding studies support that NPC1L1 is the molecular target of ezetimibe [18,19].

Abbreviations: ABC, ATP-binding cassette; BW, body weight; $L1^{LivOnly}$ mice, mice expressing no endogenous NPC1L1, but human NPC11 in liver only; NPC1L1, Niemann-Pick C1-Like 1; L1-KO, NPC1L1 knockout

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The tissue distribution of NPC1L1 expression varies among species. In rodents, NPC1L1 is almost exclusively expressed in the small intestine, but human livers also express NPC1L1 [8,20,21]. In the small intestine, NPC1L1 protein localizes at the apical surface of absorptive enterocytes facilitating absorption of cholesterol from the intestinal lumen [8,22]. We found that NPC1L1 resides at the canalicular membrane of hepatocytes in the liver of nonhuman primates and humans [21,23]. To investigate the function of liver NPC1L1 in cholesterol metabolism, we generated transgenic mice overexpressing human NPC1L1 in the liver [21]. Consistent with the localization of NPC1L1 in humans and nonhuman primates, overexpressed human NPC1L1 also localizes at the canalicular membrane of hepatocytes in the liver of transgenic mice. In the absence of altered expression of the cholesterol exporter ABCG5/G8, transgenic mice display a dramatic reduction in biliary cholesterol excretion and a significant increase in blood cholesterol, suggesting a role of hepatic NPC1L1 in regulating biliary cholesterol excretion [21]. Treatment of these transgenic mice with ezetimibe essentially restores biliary cholesterol excretion, suggesting that hepatic NPC1L1 may be a target of ezetimibe. However, since these transgenic mice express high amounts of endogenous NPC1L1 in the small intestine and ezetimibe inhibits intestinal NPC1L1, we could not distinguish the hepatic and intestinal actions of ezetimibe in our transgenic mice. To definitively establish the role of hepatic NPC1L1 in modulating cholesterol metabolism and the action of ezetimibe in liver, we crossed our liver-specific transgenic mice to L1-KO mice, and generated mice expressing no endogenous NPC1L1, but human NPC1L1 in liver ($L1^{LivOnly}$ mice). Findings from these animals definitively established that hepatic NPC1L1 inhibits biliary cholesterol excretion and is a target of ezetimibe action. Additionally, we showed that blocking biliary cholesterol excretion does not influence fecal excretion of cholesterol mass in $L1^{LivOnly}$ mice on a diet containing 0.2% cholesterol, an amount similar to that found in a typical Western type diet.

2. Materials and methods

2.1. Animals

L1-KO mice were created using C57BL/6 embryonic stem cells, thus having pure C57BL/6 genetic background [20]. Liver-specific NPC1L1 transgenic mouse founders were created using B6D2 embryos and these founders were then crossed with B6D2 mice to establish liver-specific NPC1L1 transgenic mouse lines [21]. To generate $L1^{LivOnly}$ mice and L1-KO mice with the same genetic background, L1-KO mice of pure C57BL/6 background were first crossed with liver-specific NPC1L1 transgenic mice (line L1-Tg112) of mixed genetic background (50% C57BL/6 and 50% D2 genetic background). Their offspring were heterozygous for NPC1L1 knockout allele, and those positive for human NPC1L1 transgene were subsequently crossed with L1-KO mice of pure C57BL/6 background. This breeding generated some L1-KO mice with human NPC1L1 transgene, which were then crossed to L1-KO mice of pure C57BL/6 background mice to establish two mouse lines: L1-KO and $L1^{LivOnly}$. These mice had 93.75% of C57BL/6 background. All mice were housed in a specific pathogen-free animal facility in plastic cages in a temperature-controlled room (22 °C) with a 12-h light/12-h dark cycle. The mice were fed ad libitum a cereal-based rodent chow diet unless stated otherwise, and had free access to water. All animal procedures were approved by the Animal Care and Use Committee at Wake Forest University Health Sciences.

2.2. Diets and ezetimibe treatments

At 2 months of age, male mice were fed a synthetic low-fat, high-cholesterol diet containing 10% energy from palm oil, 0.2% (w/w) cholesterol. The diet was prepared at the institutional diet core and used in many previous studies [21,24]. After being fed the diet for 18 days, the mice were treated by gavages daily on days 18–21 either with 10 mg/kg body weight (BW) ezetimibe suspended in 100 μl 0.4% methyl cellulose or with 100 μl 0.4% methyl cellulose alone (vehicle). After 4 days of vehicle or ezetimibe treatment, the mice were fasted for 4 h during the daylight cycle and then sacrificed. Blood, bile, and tissues were collected.

A subset of 6-week-old male mice on chow diet was treated with ezetimibe or vehicle for 4 days and then subject to bile duct cannulation as we have described previously [21]. The only modification was that we ligated the cystic bile duct in this study.

2.3. Analysis of lipid concentrations in plasma, bile and liver

The plasma was analyzed for total and free cholesterol, phospholipids and triglyceride by using Cholesterol/HP (Roche), Free cholesterol C (Wako), Phospholipids (Wako), and Triglycerides (Roche) enzymatic assay kits, respectively.

For analysis of hepatic lipid contents, the lipids were extracted from ~100 mg of liver tissues and quantified enzymatically as described previously [25].

Lipid concentrations in the gallbladder bile were measured as described previously [21].

2.4. Measurements of fecal excretion of neutral sterols and bile acids

After being fed the synthetic 0.2% cholesterol diet for 18 days, the mice were individually housed for 3 days. The feces were collected, dried in a 70 °C vacuum oven, weighed, and crushed into powder. A measured mass (~100 mg) of feces was placed into a glass tube containing 103 μg of 5α-cholestane as an internal standard. The feces were saponified in alcoholic KOH solution [2 ml 95% ethanol and 200 μl 50% KOH (w/v in water)] by placing the tubes in a heating block set at 70 °C for 3 h. The lipids were extracted by adding 2 ml hexane and 2 ml water. After centrifugation at 2700 rpm at room temperature for 10 min, 1 ml of hexane phase was transferred to a 2 ml gas chromatography vial. The neutral sterols were then analyzed by gas–liquid chromatography on an Agilent 6890 gas chromatograph with a cool-on-column inlet, Agilent 7683B Series auto-injector, and flame ionization detector. The column used was a ZB50 column (Phenomenex) (15 m × 0.53 mm id, 1 μm film thickness) with a 1 m × 0.53 mm id precolumn; carrier gas (hydrogen) at 6 psi head pressure, 15 ml/min at 250 °C; carrier gas + make-up gas (nitrogen) at 20 ml/min; and isothermal program at 250 °C with the detector at 280 °C. Total run time was 15 min. Neutral sterols (cholesterol, coprostanol and cholestanone) were identified by retention time comparison to standards. The mass of each sterol in the sample was calculated from peak area as follows:

$$(\textit{sterol area} \div 5\alpha\text{-}\textit{cholestane area})\ \textit{in sample} \div (\textit{sterol area} \div 5\alpha\text{-}\textit{cholestane area})\ \textit{in standard} \times (\textit{amount of } 5\alpha\text{-}\textit{cholestane added to tube} \div \text{g } \textit{feces extracted}) = \text{mg } \textit{sterol}/\text{g } \textit{feces}.$$

Fecal bile acid excretion was determined using an enzymatic assay as we have described previously [26].

2.5. Statistical analysis

All data were reported as the mean ± the standard error of the mean (SEM). The differences between the mean values of L1-KO and $L1^{LivOnly}$ groups were tested for statistical significance by the two-tailed Student's *t* test. Significant differences were determined for the values among 4 groups (vehicle or ezetimibe-treated L1-KO and