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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.

### Keywords

NPC1L1; cholesterol absorption; Zetia; transgenic



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## 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 20$^{th}$ century [3], and the key molecules responsible for hepatobiliary cholesterol secretion and intestinal cholesterol absorption have been identified at the dawn of the 21$^{st}$ 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].

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 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]. Consistently 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 two 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 10mg/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 2,700 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: (sterol area ÷ 5α-cholestane area) in sample ÷ (sterol area ÷ 5α-cholestane area) in standard × (amount of 5α-cholestane added to tube ÷ g feces extracted) = mg sterol/g feces.

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

### 2.5. Statistical analysis

All data was 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 $L1^{LivOnly}$ mice) by ANOVA (Tukey-Kramer honestly significant difference). *P* value less than 0.05 was considered significant.

## 3. RESULTS

### 3.1. Biliary cholesterol excretion is dramatically decreased in $L1^{LivOnly}$ mice

We have previously demonstrated that the human NPC1L1 protein is specifically expressed in the liver of our transgenic mice on the wild-type background [21]. In this study, we confirmed that the human NPC1L1 transgene was strongly expressed in liver, but not small intestine of $L1^{LivOnly}$ mice that had an NPC1L1 knockout background (Fig. 1A). Our previous study has also shown that the hepatic overexpression of NPC1L1 in wild-type mice blocks biliary cholesterol excretion without altering hepatic expression levels of the major hepatobiliary cholesterol exporter ABCG5/G8 [21], suggesting a direct role of NPC1L1 in modulating biliary cholesterol excretion. To clarify the role of liver NPC1L1 in biliary cholesterol secretion, we measured lipid compositions of gallbladder bile in $L1^{LivOnly}$ mice. The gallbladder cholesterol concentration was used as an indicator for biliary cholesterol excretion as previous studies have shown that it is correlated well with hepatobiliary cholesterol secretion rates in many mouse models [4, 5, 27, 28], including our NPC1L1 transgenic mice on the wild-type background [29]. After being fed the 0.2% cholesterol diet for 21 days, the gallbladder cholesterol concentration was 91% lower in $L1^{LivOnly}$ mice than L1-KO mice (0.22 μmol/ml in $L1^{LivOnly}$ mice versus 2.32 μmol/ml in L1-KO mice), while gallbladder phospholipid and bile acid concentrations remained unchanged in $L1^{LivOnly}$ mice (Fig. 1). Because the bile can be concentrated in the gallbladder, potentially altering

lipid concentrations, the molar ratio of the biliary lipids were calculated. The molar ratio of biliary cholesterol was decreased by 90% in $L1^{LivOnly}$ mice (0.13% for $L1^{LivOnly}$ versus 1.27% for L1-KO), while the molar ratio of phospholipids and bile acids were largely maintained (Fig. 1B). Thus, transgenic overexpression of human NPC1L1 in the liver of mice lacking endogenous NPC1L1 specifically inhibits biliary cholesterol excretion.

### 3.2. Fecal excretion of cholesterol and bile acids is maintained in $L1^{LivOnly}$ mice

Biliary secretion can be a significant contributor to fecal cholesterol [5, 30, 31]. We developed a mouse model that has more than 90% reduction in biliary cholesterol excretion, and therefore could have a dramatic reduction in fecal cholesterol secretion in these animals. However, the fecal excretion of neutral sterols (cholesterol and its bacterial modified metabolites coprostanol and cholestanone) in $L1^{LivOnly}$ mice was unaffected (84.3 ± 3.1 μmol/day/100g BW in $L1^{LivOnly}$ versus 88.4 ± 2.2 μmol/day/100g BW in L1-KO) (Fig. 2A). Fecal bile acid excretion was also maintained (12.4 ± 1.5 μmol/day/100g BW in $L1^{LivOnly}$ versus 9.6 ± 1.1 μmol/day/100g BW in L1-KO) (Fig. 2B). The daily stool output (g/day/100g BW) also did not differ between $L1^{LivOnly}$ and L1-KO mice (Fig. 2C). These results indicate that disruption of biliary cholesterol excretion has no effect on fecal cholesterol excretion in $L1^{LivOnly}$ mice on the 0.2% cholesterol diet, consistent with our previous study [29].

### 3.3. Hepatic cholesterol content is increased in $L1^{LivOnly}$ mice

By blocking biliary cholesterol excretion, hepatic NPC1L1 may cause cholesterol accumulation in the liver. To examine this possibility, we analyzed hepatic lipids (Table 1). After being fed the 0.2% cholesterol diet for 3 weeks, no differences were found in the hepatic contents of cholesterol ester and triglyceride between $L1^{LivOnly}$ mice and L1-KO mice, while the hepatic contents of total cholesterol, free cholesterol and phospholipids were slightly but significantly increased in $L1^{LivOnly}$ mice. Thus, inhibiting biliary cholesterol excretion modestly influences hepatic homeostasis of free cholesterol and phospholipids.

### 3.4. Plasma cholesterol concentration is elevated in $L1^{LivOnly}$ mice

Biliary excretion is a major pathway by which the body eliminates excess cholesterol. We have previously shown that blocking this pathway does not cause a dramatic accumulation of cholesterol in the liver, a suppression of hepatic genes involved in cholesterol biosynthesis, or any changes in hepatic expression levels of several major genes related to lipoprotein cholesterol metabolism, including ABCA1, scavenge receptor class B type I, and low density lipoprotein receptor in NPC1L1 transgenic mice that also express endogenous NPC1L1 in the intestine [21, 29]. We have instead observed a significant increase in plasma cholesterol in these animals [21, 29]. To determine if hepatic NPC1L1 influences blood lipid homeostasis in $L1^{LivOnly}$ mice, we measured plasma concentrations of cholesterol, triglyceride and phospholipids in $L1^{LivOnly}$ mice (Table 2). After being fed the 0.2% cholesterol diet for 21 days, $L1^{LivOnly}$ mice displayed significantly higher levels of plasma total cholesterol, free cholesterol and cholesterol ester than L1-KO mice. Plasma concentrations of phospholipids and triglyceride did not differ between $L1^{LivOnly}$ and L1-KO mice.

### 3.5. Effects of ezetimibe on cholesterol metabolism in $L1^{LivOnly}$ mice

We have previously shown that ezetimibe restores biliary cholesterol excretion in the liver-specific NPC1L1 transgenic mice expressing endogenous NPC1L1 [21], implying that the drug acts in the liver. However, ezetimibe simultaneously inhibits endogenous intestinal NPC1L1, confounding the explanation of our observations. To clarify ezetimibe action in the liver, we treated $L1^{LivOnly}$ mice with ezetimibe at 10 mg/kg BW for 4 days, collected

bile samples from gallbladders, and measured biliary lipid concentrations (Fig. 3A). Based on the molar ratio, a reduction of ~90% was observed for the molar percentage of biliary cholesterol in vehicle-treated $L1^{LivOnly}$ mice versus either vehicle-treated or ezetimibe-treated L1-KO mice. Administration of ezetimibe to $L1^{LivOnly}$ mice caused an 8.5-fold increase in the molar percentage of biliary cholesterol compared with vehicle-treated $L1^{LivOnly}$ mice. In addition, this biliary cholesterol molar ratio was not significantly different from that in either vehicle-treated or ezetimibe-treated L1-KO mice. Unlike cholesterol, the molar percentage of biliary phospholipids and bile acids did not differ between L1-KO and $L1^{LivOnly}$ mice treated with either vehicle or ezetimibe. To further validate the effects of hepatic NPC1L1 and ezetimibe on biliary lipid output, we measured bile flow rates and biliary lipid secretion rates via common bile duct cannulation in a subset of mice (L1-KO, $L1^{LivOnly}$, and $L1^{LivOnly}$ treated with ezetimibe). Remarkably, hepatic overexpression of human NPC1L1 reduced the biliary cholesterol secretion to an undetectable level, which was restored significantly by ezetimibe treatment for 4 days (Table 3). Neither hepatic overexpression of NPC1L1 nor ezetimibe treatment had a dramatic effect on bile flow, phospholipid secretion, or bile acid secretion (Table 3). These findings demonstrated that ezetimibe inhibits hepatic NPC1L1 function and specifically promotes biliary cholesterol excretion.

While a more than 90% reduction in biliary cholesterol excretion did not influence fecal excretion of cholesterol mass in $L1^{LivOnly}$ mice (Fig. 2A), we reasoned that the restoration of biliary cholesterol excretion in $L1^{LivOnly}$ mice by ezetimibe (Fig. 3A and Table 3) may increase fecal cholesterol output. Thus, we measured fecal neutral sterol excretion in $L1^{LivOnly}$ mice treated with ezetimibe. Interestingly, the restoration of biliary cholesterol excretion had no effects on fecal excretion of neutral sterol mass $L1^{LivOnly}$ mice under our experimental conditions (Fig. 3B).

To determine if ezetimibe treatment can normalize plasma cholesterol levels in $L1^{LivOnly}$ mice, we measured plasma total cholesterol concentrations in $L1^{LivOnly}$ mice and their littermate control L1-KO mice (Fig. 3C). While vehicle-treated $L1^{LivOnly}$ mice maintained a significant 38.6% increase in plasma total cholesterol concentrations compared with vehicle-treated L1-KO mice, administration of ezetimibe to $L1^{LivOnly}$ mice for 4 days did not reduce the plasma total cholesterol to the level seen in L1-KO mice treated with either vehicle or ezetimibe.

## 4. DISCUSSIONS

In this study, we created a mouse model expressing no endogenous NPC1L1, but human NPC1L1 in liver only ($L1^{LivOnly}$ mice). Using this model we demonstrated that hepatic overexpression of NPC1L1 directly inhibits biliary cholesterol excretion and increases cholesterol levels in liver and blood. In addition, we showed that ezetimibe treatment restores biliary cholesterol excretion in $L1^{LivOnly}$ mice. Thus we have definitively established that hepatic NPC1L1 is a target of ezetimibe, at least in mice.

Although the mechanism by which hepatic NPC1L1 inhibits biliary cholesterol excretion remains to be molecularly and biochemically elucidated, we have previously shown that transgenically overexpressed NPC1L1 protein resides at the canalicular membrane of hepatocytes in the liver of our transgenic mice and this genetic manipulation does not influence hepatic expression of the hepatobiliary cholesterol exporter ABCG5/G8 [21], suggesting an ABCG5/G8-independent mechanism. In humans and nonhuman primates, NPC1L1 also localizes at the canalicular membrane of hepatocytes [21, 23]. Given that intestinal NPC1L1 resides at the apical membrane of enterocytes mediating free cholesterol transport from the gut lumen into enterocytes [8, 22] and that stable overexpression of

NPC1L1 in cultured cells facilitates free cholesterol uptake [23, 32], it is conceivable that hepatic NPC1L1 may transport free cholesterol from the canalicular bile back into hepatocytes, counterbalancing ABCG5/G8-mediated hepatobiliary cholesterol secretion. This mechanism may have evolved to prevent excess loss of cholesterol from bile because cholesterol is essential for maintaining normal function of cells and substantial energy input is required to synthesize a cholesterol molecule from simple substrates.

Ezetimibe was considered to lower plasma cholesterol levels exclusively by inhibiting cholesterol absorption through targeting intestinal NPC1L1 [33]. However, the drug has the opportunity to physically interact with the hepatic NPC1L1 protein as its active glucuronide form transits through the bile canalicular membrane en route back to the intestinal lumen [33]. In this study, when $L1^{LivOnly}$ mice were treated with ezetimibe for 4 days, biliary cholesterol was largely restored. Since these animals expressed no endogenous NPC1L1, but only human NPC1L1 in liver, our observation undoubtedly established that hepatic NPC1L1 is a target of ezetimibe in mice. Despite the restoration of biliary cholesterol excretion, ezetimibe treatment for 4 days failed to reduce the plasma cholesterol concentration to normal in $L1^{LivOnly}$ mice. This result is different from our previous finding showing that administration of ezetimibe for 4 days to liver-specific NPC1L1 transgenic mice expressing endogenous NPC1L1 reduces the plasma total cholesterol concentration modestly but significantly by 26% when compared with the same mice treated with vehicle [21]. One possible explanation is that in the previous study ezetimibe inhibits the function of not only hepatic, but also intestinal NPC1L1, thereby causing this difference. Alternatively, a longer treatment may be required for ezetimibe to normalize blood cholesterol concentration in $L1^{LivOnly}$ mice.

Normally, ~50% of bile-derived free cholesterol is re-absorbed in the small intestine of humans and mice [31, 34, 35], the rest of which is lost in feces. Initially it may seem surprising that a more than 90% reduction in biliary cholesterol excretion would yield little change in fecal cholesterol excretion for the $L1^{LivOnly}$ mice (Fig. 2A). However, this is the predicted result in a model with impaired intestinal cholesterol absorption that was fed a high cholesterol containing diet. Intestinal cholesterol absorption rates play a critical role in determining how much cholesterol is excreted in feces. In the present study, this factor was minimized because $L1^{LivOnly}$ mice were used and these animals cannot efficiently absorb cholesterol as a result of deletion of NPC1L1 in the intestine [8]. For comparison to literature values, the values for cholesterol turnover are calculated as mg/day/100 g BW [36]. In this model, the dietary input of cholesterol is approximately 32 mg/day/100g BW (calculated using the average daily consumption of 4 g/day for the synthetic diet, 0.2% cholesterol (w/w), and an average of 25 g body for the mouse models) and fecal neutral sterol excretion is 34.2 mg/day/100g BW and 32.6 mg/day/100g BW in the L1-KO and $L1^{LivOnly}$ mice, respectively (Fig. 1). Assuming an average hepatic bile output of approximately 2.5 ml/day for a 25 g mouse [37] and using the measured cholesterol concentrations of hepatic bile (Table 3), the biliary output of cholesterol is calculated to be only approximately 1.35 mg/day/100g BW and 0.15 mg/day/100g BW in the L1-KO and $L1^{LivOnly}$ mice, respectively, and represents only a small fraction (< 4%) of the daily fecal neutral sterol output. Similar calculations can be made to explain why despite a significant restoration of biliary cholesterol excretion by ezetimibe (Fig. 3A and Table 3), $L1^{LivOnly}$ mice displayed no apparent increase in fecal cholesterol excretion (Fig. 3B). Since unlike intestinal and hepatic sterol transport, bile acid transport should not be directly affected by altering NPC1L1 expression, bile acid output was also calculated. Using these values for hepatic bile output and biliary bile acid secretion (Table 3), the biliary output of bile acid is calculated to be 312.5 μmol/day/100 g BW and 322.8 μmol/day/100 g BW in the L1-KO and $L1^{LivOnly}$ mice, respectively. With a daily fecal bile acid excretion of 9.6 μmol/day/100 g BW and 12.4 μmol/day/100 g BW, the percent daily recovery of bile acids from the

intestine was approximately 96.9% and 96.1% in the L1-KO and $L1^{LivOnly}$ mice, respectively, values that are in good agreement with previous measurements for bile acid turnover in mice [38, 39]. This suggests that the biliary sampling is representative of daily bile output, at least for bile acids.

The model was designed to unmask effects of hepatic NPC1L1 on biliary cholesterol secretion. In this regard, these results are important to consider with regard to humans and their susceptibility to cholesterol gallstones. In humans under basal conditions, as well as in virtually all species examined, bile is unsaturated [40]. In bile, free cholesterol is solubilized in mixed micelles containing phospholipids and bile acids. The ratio of the actual amount of cholesterol present to the maximal micellar solubility is defined as cholesterol saturation index [41]. If gallbladder cholesterol concentrations are higher than what can be solubilized in mixed micelles, cholesterol saturation indices increase and gallstones may form. Hepatobiliary hypersecretion of cholesterol is the primary pathophysiologic defect in most humans with cholesterol gallstones [42]. NPC1L1 is expressed in human livers [8, 20, 21]. Although the physiological function of NPC1L1 in human liver has yet to be examined directly in humans, the finding from our animal model suggests that hepatic NPC1L1 may counterbalance hepatobiliary cholesterol secretion mediated by ABCG5/G8 heterodimer [4–6], thereby reducing gallbladder cholesterol saturation indices and preventing cholesterol gallstone formation. Ezetimibe treatment may increase hepatobiliary cholesterol secretion and cholesterol gallstone formation through inhibiting hepatic NPC1L1. However, the drug simultaneously inhibits intestinal NPC1L1 to reduce intestinal cholesterol absorption, which will decrease the transport of cholesterol to the liver for hepatobiliary secretion. In this case, ezetimibe treatment may actually reduce cholesterol saturation indices and gallstone formation as observed in hamsters [43], mice [44], and a few human subjects [45].

In conclusion, hepatic overexpression of NPC1L1 in mice directly inhibits biliary cholesterol excretion and increases blood cholesterol. Hepatic NPC1L1 is a target of ezetimibe.

Research Highlights
The findings directly demonstrated that hepatic NPC1L1 inhibits biliary cholesterol excretion and is a target of ezetimibe.

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## 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 |

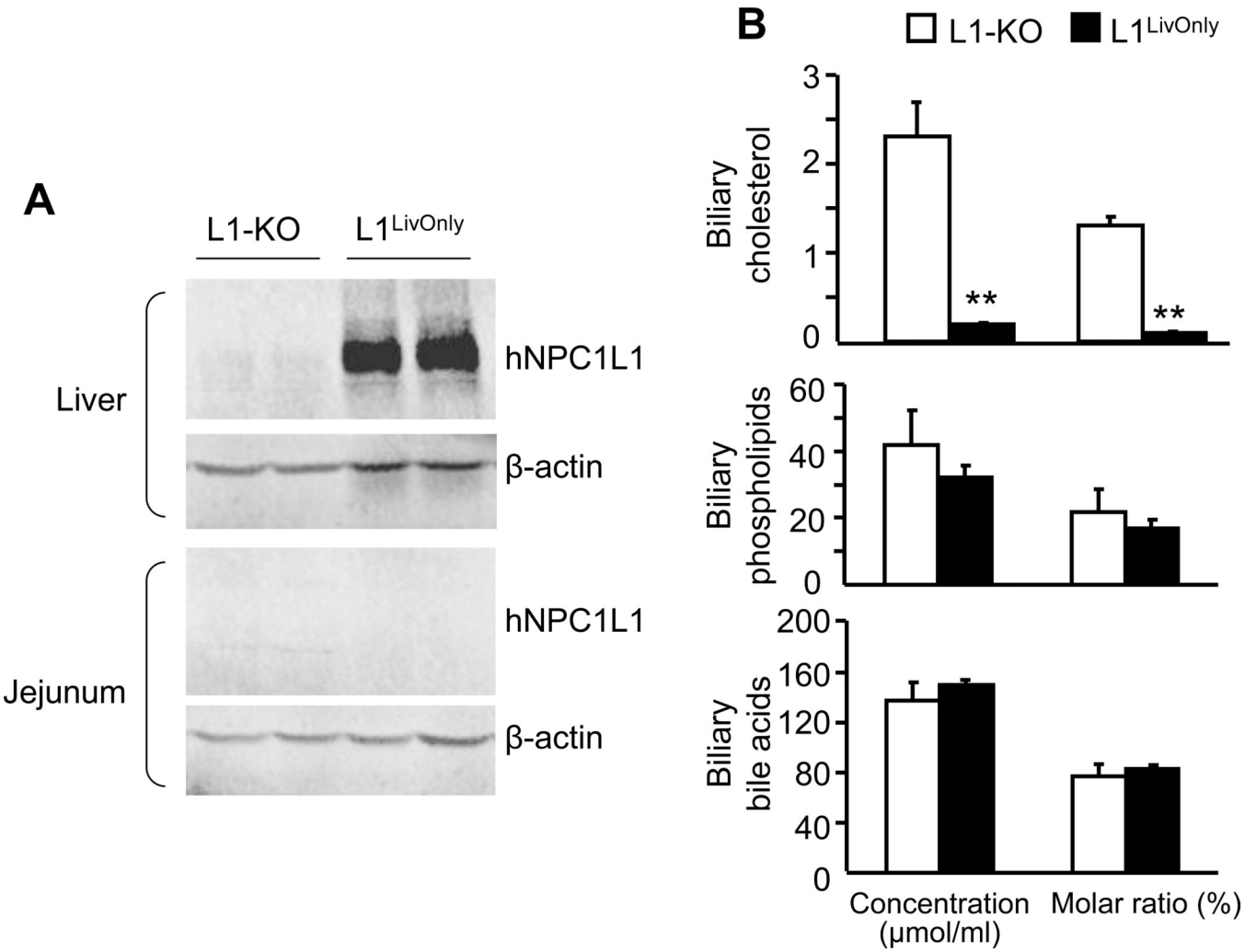


**FIG. 1.**

(A) Liver-specific expression of human NPC1L1 in the transgenic mice determined by Western blotting using an anti-human NPC1L1 antibody [21]. (B) Hepatic NPC1L1 specifically inhibits biliary cholesterol excretion in $L1^{LivOnly}$ mice. Biliary lipid concentrations in the gallbladder of L1-KO and $L1^{LivOnly}$ mice were determined as described under Materials and Methods, and lipid molar ratios were calculated. Each bar represents Mean ± SEM of 6 samples, and statistically significant differences were determined by the two-tailed Student's *t* test. $*P < 0.05$; $**P < 0.01$.

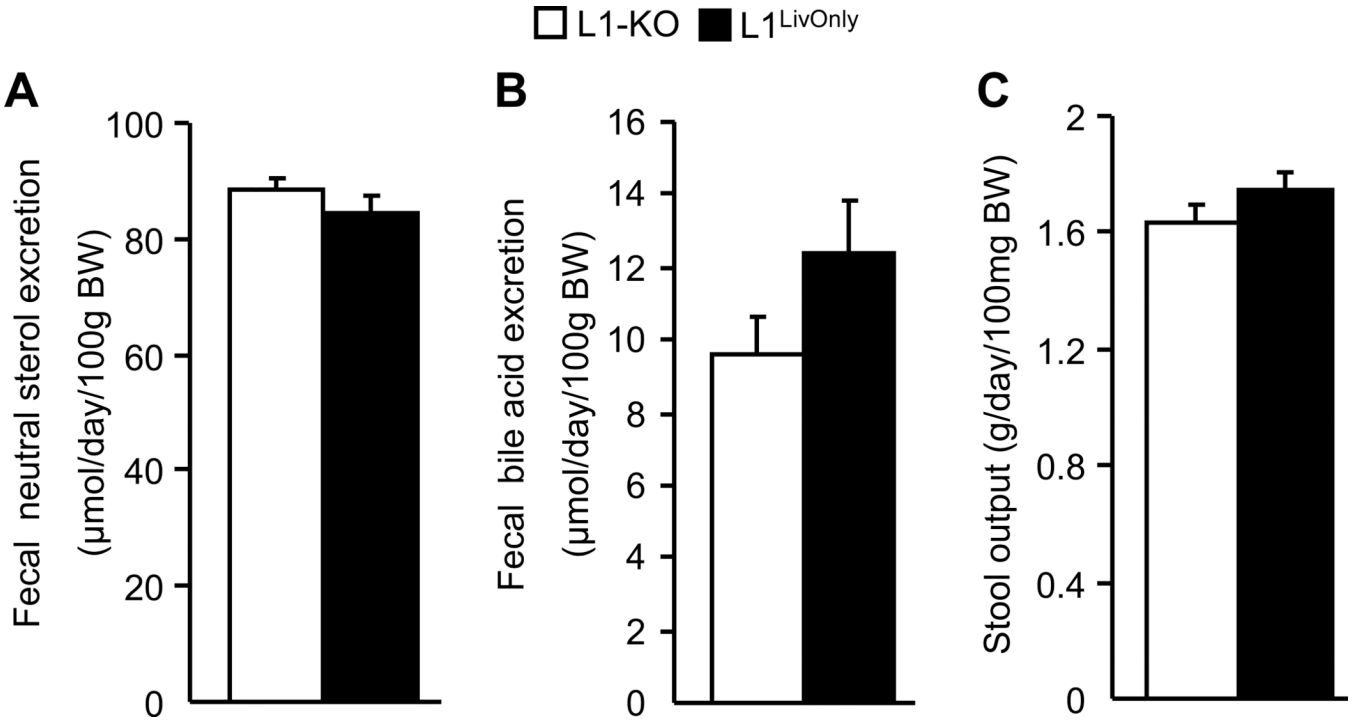


**FIG. 2.**

(A) Fecal neutral sterol excretion in $L1^{LivOnly}$ mice. The amount of neutral sterols is a sum of cholesterol and its bacterial metabolites coprostanol and cholestanone. (B) Fecal bile acid excretion in $L1^{LivOnly}$ mice. (C) Stool output. Each bar represents Mean ± SEM (n = 5–6), and statistically significant differences were determined by the two-tailed Student's *t* test.

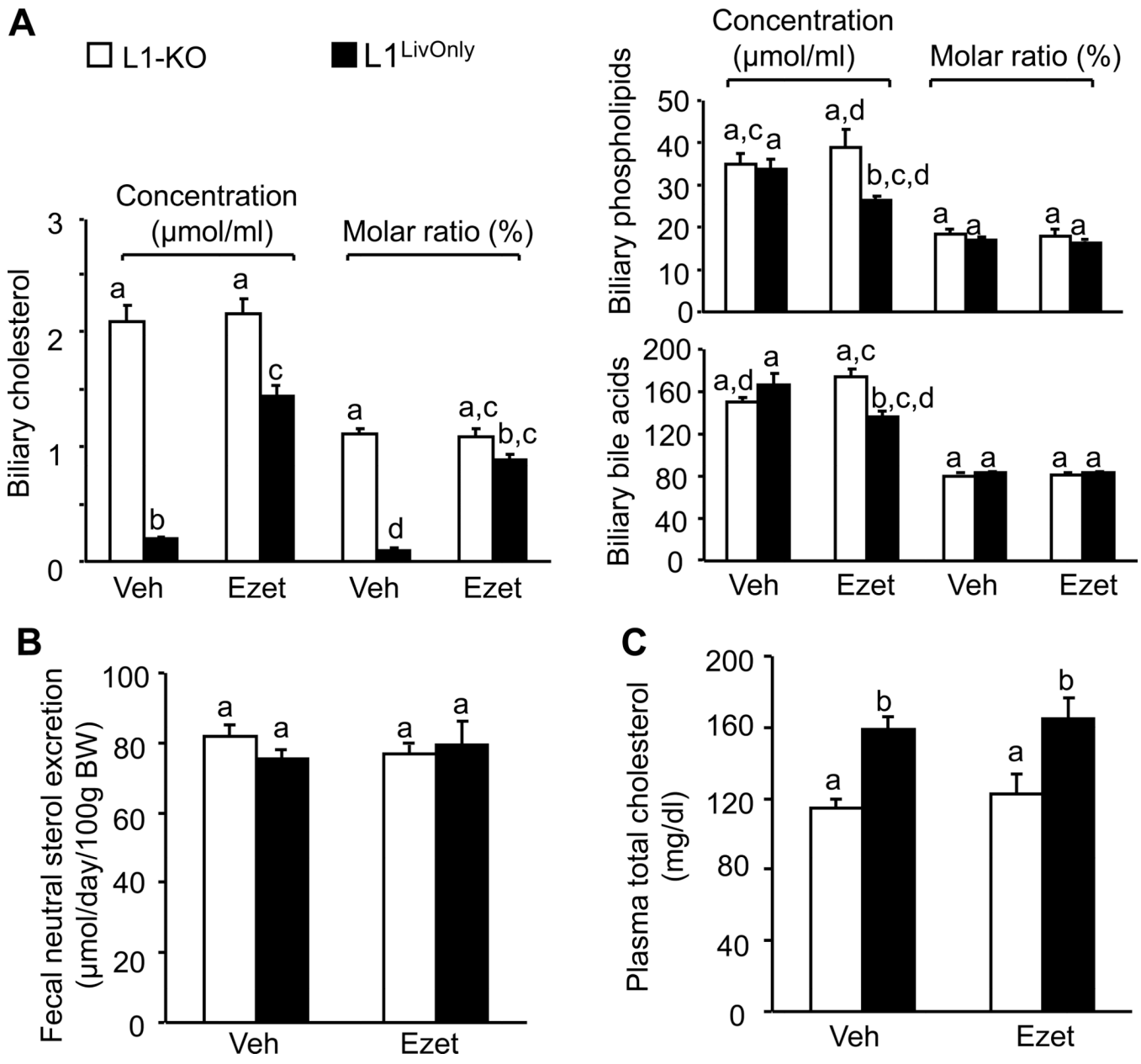


**FIG. 3.**

Ezetimibe restores biliary cholesterol concentrations in $L1^{LivOnly}$ mice. After being fed the 0.2% cholesterol diet for 18 days, L1-KO and $L1^{LivOnly}$ mice were gavaged daily on days 18–21 with either vehicle (Veh) or ezetimibe (Ezet) (10mg/kg BW/day). After a 4-h fast on day 22, mice were sacrificed. (A) Lipid concentrations and molar ratios in the gallbladder bile. (B) Fecal neutral sterol excretion. (C) Plasma total cholesterol concentrations. Values are Mean ± SEM of 5–6 samples. Statistic significance was tested by ANOVA (Tukey-Kramer honestly significant difference), and the difference between values associated with different letters is statistically significant ($P < 0.05$).

**TABLE 1**

Hepatic lipid contents

| Genotype | TC | FC | CE | PL | TG |
|---|---|---|---|---|---|
| | Mean ± SEM (mg/g wet weight) | | | | |
| L1-KO | 2.4 ± 0.05 | 2.0 ± 0.02 | 0.8 ± 0.06 | 81 ± 2 | 62 ± 7 |
| $L1^{LivOnly}$ | 2.7 ± 0.08* | 2.1 ± 0.04* | 1.0 ± 0.01 | 93 ± 3* | 57 ± 10 |

L1-KO and $L1^{LivOnly}$ mice (n = 6) were fed the synthetic 0.2% cholesterol diet for 21 days. The mice were fasted for 4 h prior to plasma collections for analyses of total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE), phospholipids (PL), and triglyceride (TG). Cholesterol ester concentrations were calculated by multiplying the difference between total and free cholesterol mass by 1.67. Statistically significant differences were determined by the two-tailed Student's *t* test.

*$P < 0.01$.

**TABLE 2**

Plasma lipid concentrations

| Genotype | TC | FC | CE | PL | TG |
|---|---|---|---|---|---|
| | Mean ± SEM (mg/dl) | | | | |
| L1-KO | 116 ± 4 | 32 ± 1 | 140 ± 5 | 244 ± 6 | 48 ± 11 |
| $L1^{LivOnly}$ | 172 ± 5* | 48 ± 1* | 207 ± 7* | 291 ± 6* | 57 ± 10 |

Hepatic total cholesterol (TC), free cholesterol (FC), cholesterol ester (CE), phospholipids (PL), and triglyceride (TG) were analyzed in individual male animals (n = 6), as described in Materials and Methods. Cholesterol ester concentrations were calculated by multiplying the difference between total and free cholesterol mass by 1.67. Statistically significant differences were determined by the two-tailed Student's *t* test.

*$P < 0.05$.

## TABLE 3

Bile flow and lipid secretion rates of L1-KO mice, $L1^{LivOnly}$ mice, and $L1^{LivOnly}$ mice treated with ezetimibe.

| | L1-KO | $L1^{LivOnly}$ | $L1^{LivOnly}$+ezet. |
|---|---|---|---|
| Bile flow (μl/min/100 g BW) | 7.2 ± 0.6 | 5.7 ± 0.4 | 6.2 ± 1.0 |
| Cholesterol (nmol/min/100 g BW) | 2.5 ± 0.2 | UD | 0.9 ± 0.1* |
| Phospholipids (nmol/min/100 g BW) | 51 ± 2 | 35 ± 3* | 37 ± 8 |
| Bile acids (nmol/min/100 g BW) | 255 ± 23 | 184 ± 43 | 314 ± 144 |

Six-week-old chow-fed male mice received by daily gavage either 10 mg/kg body weight (BW) ezetimibe (ezet.) suspended in 100 μl of 0.4% methyl cellulose, or 100 μl of 0.4% methyl cellulose alone (vehicle). After 4 days of ezetimibe or vehicle treatment, the common bile duct was cannulated and bile samples were collected for 10 min for the analysis of lipids as described under the Methods. Statistically significant differences were determined by the two-tailed Student's *t* test.

*$P < 0.01$ (versus the respective value in L1-KO group). UD, undetectable.