Ezetimibe Inhibits Hepatic Niemann-Pick C1-Like 1 to Facilitate Macrophage Reverse Cholesterol Transport in Mice

Ping Xie, Lin Jia, Yinyin Ma, Juanjuan Ou, Hongming Miao, Nanping Wang, Feng Guo, Amirfarbod Yazdanyar, Xian-Cheng Jiang, Liqing Yu

Objective—Controversies have arisen from recent mouse studies about the essential role of biliary sterol secretion in reverse cholesterol transport (RCT). The objective of this study was to examine the role of biliary cholesterol secretion in modulating macrophage RCT in Niemann-Pick C1-Like 1 (NPC1L1) liver only (L1LivOnly) mice, an animal model that is defective in both biliary sterol secretion and intestinal sterol absorption, and determine whether NPC1L1 inhibitor ezetimibe facilitates macrophage RCT by inhibiting hepatic NPC1L1.

Approach and Results—L1LivOnly mice were generated by crossing NPC1L1 knockout (L1-KO) mice with transgenic mice overexpressing human NPC1L1 specifically in liver. Macrophage-to-feces RCT was assayed in L1-KO and L1LivOnly mice injected intraperitoneally with [3H]-cholesterol–labeled peritoneal macrophages isolated from C57BL/6 mice. Inhibition of biliary sterol secretion by hepatic overexpression of NPC1L1 substantially reduced transport of [3H]-cholesterol from primary peritoneal macrophages to the neutral sterol fraction in bile and feces in L1LivOnly mice without affecting tracer excretion in the bile acid fraction. Ezetimibe treatment for 2 weeks completely restored both biliary and fecal excretion of [3H]-tracer in the neutral sterol fraction in L1LivOnly mice. High-density lipoprotein kinetic studies showed that L1LivOnly mice compared with L1-KO mice had a significantly reduced fractional catabolic rate without altered hepatic and intestinal uptake of high-density lipoprotein–cholesterol ether.

Conclusions—In mice lacking intestinal cholesterol absorption, macrophage-to-feces RCT depends on efficient biliary sterol secretion, and ezetimibe promotes macrophage RCT by inhibiting hepatic NPC1L1 function. (Arterioscler Thromb Vasc Biol. 2013;33:920-925.)

Key Words: biliary cholesterol secretion ■ ezetimibe ■ fecal neutral sterol excretion ■ NPC1L1 reverse cholesterol transport ■ transgenic

Reverse cholesterol transport (RCT) is classically defined as the movement of cholesterol from cells in peripheral tissues to circulating high-density lipoproteins (HDLs) for hepatic uptake, biliary secretion, and fecal disposal. This process is believed to explain, at least in part, why increased plasma HDL-cholesterol is atheroprotective. Recently, an intestinal route for mass cholesterol excretion in the feces has been reported, which has promoted studies of the significance of this nonbiliary route in RCT, using genetic and surgical mouse models deficient in biliary cholesterol secretion.

One genetic model used in macrophage RCT assays is the transgenic mice specifically overexpressing human Niemann-Pick C1-Like 1 (NPC1L1) in liver. Unlike mice lacking the ATP-binding cassette (ABC) transporter B4 (ABCB4), which are deficient in biliary secretion of both cholesterol and phospholipids as well as develops liver cholestasis, NPC1L1 liver transgenic mice exhibit reduced biliary cholesterol secretion without showing signs of liver cholestasis. NPC1L1 is almost exclusively expressed in the small intestine of mice, and its knockout in mice blocks intestinal cholesterol absorption. The function of NPC1L1 can be inhibited by ezetimibe, a potent cholesterol absorption inhibitor, developed to lower blood cholesterol. In humans, NPC1L1 is expressed in liver in addition to intestine. We have previously shown that transgenic overexpression of human NPC1L1 in the mouse liver significantly reduces biliary cholesterol secretion without altering hepatic expression of the heterodimeric hepatobiliary cholesterol exporters, ABCG5 and ABCG8, and this reduction in biliary cholesterol secretion can be rescued by ezetimibe treatment.

Using in vivo RCT assay protocol of Rader, Temel et al showed that biliary sterol secretion is not required for macrophage RCT in NPC1L1 liver transgenic mice, and in

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mice with acute biliary diversion, both mouse models were deficient in biliary sterol secretion into the gut lumen. In striking contrast with this finding, Nijstad et al.\(^7\) reported almost simultaneously that biliary cholesterol secretion is required for functional RCT in mice using the similar protocol. They also showed that bile duct ligation in mice or genetic inhibition of biliary sterol secretion in ABCB4 knockout mice dramatically reduces macrophage-to-feces RCT. Furthermore, they showed that pharmacological stimulation of macrophage RCT by a liver X receptor agonist depends on efficient biliary sterol secretion in mice. The mechanistic basis for different conclusions in these 2 studies is unclear.

On average, \(\approx 50\%\) of cholesterol in the gut lumen is absorbed in humans and rodents,\(^{17,18}\) and the remainder excreted in feces. Inhibiting intestinal cholesterol absorption by ezetimibe has been shown to dramatically increase macrophage RCT in wild-type mice,\(^{19,20}\) a model that does not express NPC1L1 in liver.\(^1\) Altered biliary cholesterol secretion was reported to influence intestinal cholesterol absorption rates.\(^{21,22}\) Acute biliary diversion or bile duct ligation reduces intestinal cholesterol absorption and profoundly alters intestinal metabolism, including increases in intestinal cholesterol synthesis.\(^{23,24}\) To eliminate effects of cholesterol absorption changes on fecal excretion of bile-derived cholesterol, we crossed cholesterol absorption–deficient NPC1L1 knockout (L1-KO) mice\(^10\) to liver-specific NPC1L1 transgenic mice\(^6\) and generated mice expressing no endogenous NPC1L1, but human NPC1L1 in liver only (L1LivOnly mice).\(^25\) We have previously shown that ezetimibe treatment increases biliary sterol excretion by inhibiting hepatic NPC1L1\(^8,25\) This observation raised an interesting question as follows: can ezetimibe facilitate macrophage RCT by inhibiting hepatic NPC1L1? L1LivOnly mice provided us a unique opportunity to address this question. In the present study, we performed macrophage RCT assays in L1LivOnly mice using the mouse primary peritoneal macrophages. We found that the macrophage-to-feces RCT was dramatically reduced in L1LivOnly mice. The reduction in macrophage RCT in these animals was completely restored by ezetimibe treatment.

Materials and Methods
Materials and Methods are available in online-only Supplement.

Results

Hepatic Overexpression of NPC1L1 Inhibits Biliary Cholesterol Secretion and Increases Cholesterol Levels in Plasma and Liver of L1-KO Mice

In a recent study using L1LivOnly mice, we found that liver-specific overexpression of human NPC1L1 in mice of NPC1L1 knockout background almost abolished biliary cholesterol secretion, as evidenced by results from bile duct cannulation studies, and significantly increased plasma and hepatic cholesterol levels.\(^25\) Consistently, in the present study using mice of the same genotypes, we found that overexpression of human NPC1L1 in the L1-KO liver remarkably reduced biliary cholesterol concentrations and molar ratios (Figure 1A), without significantly altering biliary concentrations and molar ratios of phospholipids (Figure 1B) and bile acids (Figure 1C). The effect of hepatic NPC1L1 on biliary cholesterol was completely reversed by ezetimibe treatment for 2 weeks (Figure 1A). Ezetimibe treatment had no effects on biliary concentrations and molar ratios of phospholipids and bile acids (Figure 1B and 1C). In contrast to previous studies using the same liver-specific NPC1L1 transgenic mice on the wild-type background,\(^4\) or on NPC1L1 knockout background, but on a 0.2% cholesterol diet (\(\approx 10\) times higher than that used in the current study), we observed a significant 35.1% reduction in fecal excretion of neutral sterols (a sum of cholesterol and its bacterial metabolites coprostanol and cholestanone) in L1LivOnly compared with L1-KO mice on the 0.015% cholesterol diet (Figure 1D). This observation suggests the importance of the use of low-cholesterol diet in delineating the role of biliary sterol secretion in fecal sterol excretion in rodents. Interestingly, ezetimibe treatment for 2 weeks in L1LivOnly mice significantly increased fecal neutral sterol excretion by 46.1%.

![Figure 1](http://atvb.ahajournals.org/)

**Figure 1**. Ezetimibe treatment restores biliary cholesterol output in L1LivOnly mice. Lipid concentrations and molar ratios in the gallbladder bile of L1-KO mice (n=5), L1LivOnly mice (n=6), and L1LivOnly mice treated with ezetimibe (L1LivOnly&Eze; n=4). A, Biliary cholesterol. B, Biliary phospholipids. C, Biliary bile acids. D, Fecal neutral sterol excretion in L1-KO (n=8), L1LivOnly (n=9), and L1LivOnly&Eze (n=7) mice. *P*<0.05 between a and b groups for each measurement (ANOVA).

| Table. Lipid Contents in Plasma (mg/dL) and Liver (µg/mg Proteins; Mean±SEM) |
|-------------------|-------------------|-------------------|
|                   | L1-KO             | L1LivOnly         | L1LivOnly&Eze     |
| Plasma TC         | 209±4\(^a\)       | 274±8\(^a\)       | 222±10\(^b\)      |
| Plasma FC         | 44±3\(^a\)        | 72±2\(^a\)        | 56±4\(^a\)        |
| Plasma CE         | 257±6\(^a\)       | 315±10\(^a\)      | 259±10\(^b\)      |
| Hepatic TC        | 39.0±2.2\(^a\)    | 47.6±2.0\(^a\)    | 40.5±1.5\(^b\)    |
| Hepatic FC        | 26.5±1.6\(^a\)    | 31.6±1.6\(^a\)    | 25.8±1.3\(^b\)    |
| Hepatic CE        | 21.1±1.7\(^a\)    | 26.9±1.4\(^b\)    | 24.7±1.8\(^b\)    |

L1-KO mice, L1LivOnly mice, and L1LivOnly mice treated with ezetimibe (L1LivOnly&Eze; n=7–9 mice per group) were fasted for 4 hours during the daytime cycle before collections of blood and liver for analyses of plasma and liver contents of TC, FC, and CE. CE contents were calculated by multiplying the difference between TC and FC mass by 1.67. *P*<0.05 among a, b, c groups for each measurement (ANOVA). CE indicates cholesterol ester; FC, free cholesterol; and TC, total cholesterol.
Mice Increases Plasma and Tissue [3H]-Tracer Levels during In Vivo Macrophage RCT Studies. Consistent with elevated plasma cholesterol in L1LivOnly compared with L1-KO mice at 6, 24, and 48 hours after peritoneal injection of [3H]-cholesterol–labeled primary macrophages, which was almost completely restored in the ezetimibe-treated L1LivOnly mice (3.03±0.20% of dose/mL; Figure 4A). Ezetimibe treatment had no effects on biliary [3H]-cholesterol output in L1-KO mice (3.11±0.13% of dose/mL; Figure 4A), consistent with L1-KO mice being ezetimibe-insensitive.10 The biliary [3H]–bile acid output was comparable among 4 groups (Figure 4B). The fecal excretion represents the final step of RCT. We measured fecal excretion of [3H]-tracer in the neutral and acidic sterol fractions in mice after peritoneal injection of labeled mouse primary macrophages. Transgenic overexpression of human NPC1L1 in the liver of L1-KO mice dramatically reduced fecal [3H]–neutral sterol excretion by 40% (4.04±0.45% of dose/d per 100 g BW in L1LivOnly mice compared with 10.09±0.42% of dose/d per 100 g BW in L1-KO mice), which was virtually reversed by ezetimibe treatment for 2 weeks (a significant 3-fold increase to 10.13±1.31% of dose/d per 100 g BW; Figure 4C). Ezetimibe treatment did not alter fecal [3H]-neutral sterol excretion in L1-KO mice (9.98±0.51% of dose/d per 100 g BW; Figure 4C). Hepatic overexpression of human NPC1L1 or its inhibition by ezetimibe did not change fecal [3H]–bile acid excretion in L1-KO mice (Figure 4D), which was in agreement with our previous finding that the mass bile acid excretion via feces is similar between L1-KO and L1LivOnly mice.25

**Ezetimibe Treatment Restores Hepatic NPC1L1-Induced Inhibition of Biliary and Fecal [3H]–Cholesterol Excretion in L1LivOnly Mice**

To determine whether hepatic NPC1L1 overexpression–mediated inhibition of biliary cholesterol secretion influences macrophage-to-feces RCT in mice lacking endogenous NPC1L1 (deficient in intestinal cholesterol absorption), we measured [3H]-tracer concentrations in bile and excretion in feces in our models. We have previously shown that L1LivOnly mice were deficient in biliary secretion of cholesterol mass.25 Consistently, biliary [3H]–cholesterol output was dramatically reduced by 72.7% in L1LivOnly mice (0.84±0.07% of dose/mL) compared with L1-KO mice (3.08±0.19% of dose/mL) at 48 hours after peritoneal injection of [3H]-cholesterol–labeled primary macrophages, which was almost completely restored in the ezetimibe-treated L1LivOnly mice (3.03±0.20% of dose/mL; Figure 4A). Ezetimibe treatment had no effects on biliary [3H]-cholesterol output in L1-KO mice (3.11±0.13% of dose/mL; Figure 4A), consistent with L1-KO mice being ezetimibe-insensitive.10 The biliary [3H]–bile acid output was comparable among 4 groups (Figure 4B).

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**Hepatic Overexpression of NPC1L1 in L1-KO Mice Increases Plasma and Tissue [3H]-Tracer Levels**

To measure macrophage RCT, we first determined how hepatic NPC1L1 overexpression influences homeostasis and distribution of [3H]-cholesterol derived from intraperitoneally injected primary macrophages that were isolated from the peritoneal cavity of C57BL/6 mice and loaded with radio-labeled cholesterol. Plasma and tissue levels of [3H]-tracer during macrophage RCT studies were measured in each group (n=4) was pooled. The same amount of pooled plasma was subjected to FPLC for monitoring lipoprotein–cholesterol distribution. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein.

Hepatic overexpression of human NPC1L1 in L1-KO mice on the low-cholesterol diet increased plasma and hepatic levels of total cholesterol, free cholesterol, and cholesterol ester (Table). The increase of blood cholesterol was mainly distributed in the large HDL fraction (Figure 2), consistent with our previous study showing that increased blood cholesterol is in the apolipoprotein E–rich HDL fraction. Ezetimibe treatment for 2 weeks virtually reversed all of these alterations.

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![Figure 2. Plasma lipoprotein-cholesterol profile in L1-KO mice, L1LivOnly mice, and L1LivOnly mice treated with ezetimibe (L1LivOnly&Eze). An equal amount of plasma from each mouse in each group (n=4) was pooled. The same amount of pooled plasma was subjected to FPLC for monitoring lipoprotein-cholesterol distribution. HDL indicates high-density lipoprotein; LDL, low-density lipoprotein; and VLDL, very low-density lipoprotein.](image-url)
Hepatic Overexpression of NPC1L1 Does Not Affect Hepatic and Intestinal Uptake of [3H]Cholesteryl Oleyl Ether–Labeled HDL in L1-KO Mice

The transport of cholesterol from peripheral tissues to HDL particles is the first step of RCT. Alterations in HDL turnover and hepatic/intestinal uptake thus have the potential to influence fecal disposal of cholesterol. To determine whether hepatic overexpression of NPC1L1 affects HDL turnover and tissue uptake, we injected [3H]Cholesteryl Oleyl Ether (1H)CEt-HDL into our mice via the tail vein, and then followed the plasma decay of radioactivity. Hepatic and intestinal uptake was assessed 48 hours after injection. A, [3H]-cholesterol recovery in the gallbladder bile. B, [3H]-bile acid recovery in the gallbladder bile. C, [3H]-cholesterol recovery in the feces. D, [3H]-bile acid recovery in the feces. P<0.05 between a and b groups for each measurement (ANOVA).

Discussion

In this study, we have shown that after peritoneal injection of [3H]-cholesterol–labeled mouse primary peritoneal macrophages, L1-KO mice expressing hepatic NPC1L1 (L1LivOnly) accumulated more [3H]-tracer in blood and tissues and secreted significantly reduced amounts of [3H]-neutral sterols in gallbladder bile and feces, when compared with L1-KO mice expressing no hepatic NPC1L1. Ezetimibe treatment reversed the accumulation of [3H]-tracer in blood and tissues and restored biliary and fecal excretion of [3H]-neutral sterols in L1LivOnly mice. Our results demonstrate an essential role of biliary sterol secretion in mediating macrophage-to-feces RCT in mice deficient in intestinal cholesterol absorption. Given that human livers express NPC1L1,8,10,15 our findings suggest that ezetimibe may have a previously unappreciated action: promoting macrophage RCT via direct inhibition of hepatic NPC1L1 in humans.

Recent studies on mice genetically or surgically deficient in biliary cholesterol secretion have shown that fecal excretion of mass cholesterol is independent of biliary cholesterol secretion; therefore, the transintestinal cholesterol efflux concept was developed as an alternative explanation for these observations.3,5 This concept was subsequently used to challenge the classic view on RCT, and it was hypothesized that macrophage RCT may occur via the intestinal route.6,26 Although the study performed by Temel et al6 using the liver-specific NPC1L1 transgenic mice on the wild-type background, or using bile-diverted mice, seems to support the nonbiliary route hypothesis on RCT, the opposite conclusion was obtained from a study performed by Nijstad et al7 using ABCB4 knockout mice, scavenger receptor class B type I knockout mice, mice treated with the liver X receptor, and mice that were subjected to bile duct ligation. Our present work is in agreement with the conclusion of Nijstad et al7 that “biliary cholesterol secretion represents the major pathway relevant for RCT” in mice, but in contrast with the finding by Temel et al.6 Although the same liver-specific NPC1L1 transgenic mice were used in the present work and in the study of Temel et al, it should be emphasized that there was a fundamental difference between the 2 animal models. The present study used the liver-specific NPC1L1 transgenic mice that are deficient in intestinal cholesterol absorption as a result of NPC1L1 knockout, whereas Temel et al used the liver-specific NPC1L1 transgenic mice that have
normal cholesterol absorption attributable to the presence of endogenous NPC1L1 in the intestine. On average, ≈50% of cholesterol in the intestinal lumen is absorbed. This would suggest that ≈50% of bile-derived cholesterol is reabsorbed without loss in the feces when NPC1L1 is present. In the present study, we eliminated this reabsorption factor that has been shown to dramatically inhibit macrophage RCT. Thus, we believe our animal model is a more reliable system compared with the model used by Temel for examining how biliary cholesterol secretion modulates in vivo RCT. The data from our model clearly demonstrate that biliary cholesterol secretion is essential for macropage RCT, thereby supporting the classic view on RCT. In addition, our data strongly argue against a role of the transintestinal cholesterol efflux pathway in promoting macropage RCT, at least in our animal model.

We have previously shown that hepatic overexpression of human NPC1L1 inhibits biliary cholesterol secretion and increases plasma cholesterol without altering hepatic expression of many proteins involved in liver cholesterol homeostasis, including several cholesterol transporters (ABCG5, ABCG8, and ABCA1), HDL receptor scavenger receptor class B type I, and low-density lipoprotein receptor. We have also shown that hepatic NPC1L1 inhibits biliary cholesterol secretion in L1-KO only mice. Consistently, the present work showed that hepatic overexpression of human NPC1L1 inhibited biliary [3H]-cholesterol excretion (Figure 4A) and raised blood/tissue [3H]-tracer concentrations (Figure 3). Ezetimibe treatment completely reversed blood/tissue [3H]-tracer accumulation in L1-KO only mice (Figure 3).

Ezetimibe was developed to inhibit intestinal cholesterol absorption to lower blood cholesterol. In our previous studies, we found that the drug also inhibits hepatic NPC1L1 function to promote biliary cholesterol secretion. The present work showed that ezetimibe increases macropage-to-feces RCT by inhibiting hepatic NPC1L1. It has been previously reported by others that ezetimibe treatment stimulates macropage RCT in wild-type mice. Ezetimibe treatment completely reversed blood/tissue [3H]-tracer accumulation in L1-KO only mice (Figure 3).

In conclusion, biliary sterol secretion plays a key role in promoting macropage-to-feces RCT. Ezetimibe promotes macropage RCT via inhibiting hepatic NPC1L1 function to stimulate biliary cholesterol secretion, at least, in mice. Additionally, the data from our animal model strongly argue against a role of the transintestinal cholesterol efflux pathway in promoting macropage RCT.
monkey through the identification of the active metabolites of SCH48461. 


Reverse cholesterol transport (RCT) is classically viewed as cholesterol movement from peripheral tissues or cells, such as macrophages, to feces via biliary secretion. Recently, a transintestinal cholesterol efflux pathway was proposed to promote RCT, challenging the classical view of RCT. However, published data on this topic are very controversial because of lack of an ideal animal model. Here, we created a novel genetically altered mouse model to address this issue. Our model minimized many confounding factors affecting RCT assays. Data from this model strongly argue against a role of transintestinal cholesterol efflux and support the classical view in RCT. Additionally, our findings are the first to demonstrate that liver NPC1L1 inhibits macrophage RCT, and that ezetimibe can inhibit liver NPC1L1 to promote macrophage RCT. Given a critical role of macrophage RCT in atheroprotection and the worldwide use of ezetimibe as a cholesterol-lowering drug, the impact of our findings on human health is substantial.
Ezetimibe Inhibits Hepatic Niemann-Pick C1-Like 1 to Facilitate Macrophage Reverse Cholesterol Transport in Mice

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Materials and Methods

Mice and Diets

L1\textsuperscript{LivOnly} mice\textsuperscript{1} were generated by crossing L1-KO mice\textsuperscript{2} with liver-specific NPC1L1 transgenic mice.\textsuperscript{3} The genetic background of L1\textsuperscript{LivOnly} and control mice was 93.75% C57BL/6. All mice were housed in a specific pathogen-free animal facility in plastic cages at 22°C, with a daylight cycle from 6 AM to 6 PM. The mice were provided with water and standard chow diet (Prolab RMH 3000; LabDiet, Brentwood, MO) \textit{ad libitum}, unless stated otherwise. All animal procedures were approved by the Institutional Animal Care and Use Committee at Wake Forest University Health Sciences and at University of Maryland.

At 6 weeks of age, male L1-KO and L1\textsuperscript{LivOnly} mice were fed a synthetic diet containing 10% energy from palm oil and 0.015% (w/w) cholesterol. The diet was prepared at the institutional diet core and used in our previous studies.\textsuperscript{8} After being fed the diet for 14 days, a subgroup of L1\textsuperscript{LivOnly} mice were switched to the same diet supplemented with 0.005% (w/w) of ezetimibe.

In Vivo Macrophage RCT Studies

The \textit{in vivo} macrophage-to-feces RCT assay was performed according to the protocol developed by Rader and colleagues.\textsuperscript{4} Thioglycollate-elicited peritoneal macrophages isolated from adult wild-type C57BL/6 mice were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 µg/ml streptomycin. The cells were loaded with [\textsuperscript{3}H]-cholesterol for 24 h in 10% FBS-
containing DMEM medium supplemented with a mixture of 5 µCi/ml [³H]-cholesterol and 100 µg/ml acetylated low-density lipoprotein (LDL). Acetylated LDL converted peritoneal macrophages to lipid-laden macrophage foam cells. These cells were washed twice with PBS and equilibrated for an additional 12 h period in serum-free RPMI-1640 containing 0.2% BSA. After equilibration, the cells were harvested and resuspended in serum-free DMEM containing 0.2%BSA immediately before injection. [³H]-cholesterol labeling efficiency was measured by extracting lipids from an aliquot of cells using the method of Bligh and Dyer⁵ and by separating extracted lipids on thin layer chromatography using a solvent system (70:30:1; hexane:diethyl ether:acetic acid).

The mice were individually housed on wire bottom cages with ad libitum access to food and water, and then intraperitoneally injected with 500 µl of the cell suspension (~3x10⁶ cells/ml at ~2x10⁶ dpm/ml). Feces were collected for 48 h. At 6 h and 24 h post injection, blood samples were collected via the submandibular vein. At 48 h post injection, the mice were sacrificed to collect blood, bile, liver, small intestine, lung and spleen for analysis.

[³H]-Tracer Measurements in Bile, Feces, and Tissues

Lipids in ~10 µl of gallbladder bile from each mouse were extracted by sequentially adding/vortexing 1 ml H₂O, 3 ml methanol:chloroform (2:1), 2 ml chloroform, and 1 ml H₂O in a 16x100 mm glass tube. To separate aqueous and organic phases, the tube was centrifuged at 2,700 rpm for 10 min. The upper aqueous phase contained [³H]-bile acids, and the lower organic phase contained [³H]-cholesterol. A known volume of each phase was dried under N₂ and resuspended in 5 ml scintillation cocktail (Bio-Safe II,
Order#: 111195, Research Product International Corp., Mount Prospect, IL) for the determination of biliary recoveries of \[^3\text{H}\]-bile acids and \[^3\text{H}\]-cholesterol. The data were expressed as the percentage of \[^3\text{H}\]-tracer recovered from the total dpm injected.

Feces were collected for 48 h after injection, dried at 70°C in a vacuum oven overnight, and then weighed. The entire fecal sample from each mouse was rehydrated in 20 ml of 95% ethanol. A total of 2 ml rehydrated fecal sample was transferred into a new glass tube and saponified by adding 400 µl of 10N NaOH and heating on a 60°C heating block for 2 h. Lipids in saponified fecal sample were extracted by 6 ml hexane for 3 times. All hexane phase was dried down under N\(_2\) and the lipid extract was resuspended in scintillation cocktail for the determination of \[^3\text{H}\]-cholesterol recovery. The remaining saponified fecal sample in aqueous phase was acidified by adding ~200 µl concentrated HCl to adjust pH to <1, and then extracted with 6 ml hexane for 3 times. All hexane phase was dried down under N\(_2\) and the extract was resuspended in 5 ml scintillation cocktail for the determination of fecal \[^3\text{H}\]-bile acid recovery.

Liver, small intestine, lung, and spleen were collected from each mouse and the organ weight was recorded. The small intestine was equally separated into 5 segments. A piece of liver, each segment of small intestine, and the whole lung and spleen were placed in 16x100 mm glass tubes and extracted in 9 ml of chloroform:methanol (2:1) until the tissue sank to the bottom of the glass tube (indicative of complete extraction of lipids). After centrifugation at 2,700 rpm for 10 min, an aliquot of 5 ml of chloroform:methanol extract was dried down under N\(_2\) and resuspended in 5 ml scintillation cocktail for the determination of \[^3\text{H}\]-cholesterol recovery in each tissue.
Lipid Analyses in Plasma, Liver and Bile

Plasma concentrations of total cholesterol, free cholesterol, and triglyceride were analyzed by enzymatic assay as described previously.\(^6\) For analysis of hepatic lipid contents, the lipids were extracted from \(\sim 80\) mg of liver tissues and measured enzymatically as described previously.\(^3\) Biliary concentrations of free cholesterol, phospholipids and bile acids were determined as described previously.\(^3\)

Measurements of Fecal Neutral Sterol Excretion

The mice were individually housed. The feces were collected for 48 h and dried in a 70\(^\circ\)C vacuum oven. The dried feces were weighed and crushed. About 100 mg of feces were placed into a 16x100 mm glass tube containing 100 \(\mu\)g of 5\(\alpha\)-cholestane as an internal standard. The feces were saponified in 2 ml of 95\% ethanol and 200 \(\mu\)l of 50\% KOH (w/v in water) on a 65\(^\circ\)C heating block for 2 h. The lipids were extracted by adding 2 ml hexane and 2 ml H\(_2\)O. After centrifugation at 2,700 rpm for 10 min at room temperature, 1 ml of hexane phase was transferred to a gas chromatography vial for the determination of neutral sterols (cholesterol and its bacterial metabolites coprostanol and cholestanone) by gas-liquid chromatography.

In Vivo HDL Turnover Studies

HDL was isolated from wild-type mice and labeled with \([^3\text{H}]\text{cholesteryl oleyl ether}\) exactly as described previously.\(^7\) \([^3\text{H}]\text{CEt-HDL}\) was dialyzed with PBS and radioactivity was then counted. \([^3\text{H}]\text{CEt-HDL}\) solution (0.5 million of dpm) was injected into each mouse via the tail vein. After injection, blood was taken from the tail vein at 5 min, 30
min, 1 h, 3 h, 8 h, 12 h, 24 h, and 48 h. Plasma decay curves were generated by dividing the plasma radioactivity at each time point by the radioactivity at the initial 5 min time point after $[^3]$HCEt-HDL injection as described by Nijstas E, et al.\(^8\) The fractional catabolic rate (FCR) was calculated from the decay curves according to the Matthews method.\(^9\) The organ uptake of $[^3]$HCEt-HDL was assessed 48 h after injection and the value was expressed as a percentage of the injected dose calculated by multiplying the initial plasma counts (5 min) with the estimated plasma volume (3.5% of total body weight).\(^8\)

### Statistical Analysis

All data are presented as Mean ± SEM (Standard Error of Mean). Significance of differences was determined for each group of values by One-way ANOVA (Tukey-Kramer honestly significant difference). A $P$ value less than 0.05 was considered significant.

### References


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Ezetimibe는 간의 Niemann-Pick C1-Like 1을 억제하여 대식구의 콜레스테롤 역수송을 촉진한다.

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Summary

배경
최근 생쥐모델을 이용한 연구 중 콜레스테롤 역수송에서 담즙에서의 스테롤 분비가 필수적 역할을 하는가에 대해 논란이 있어 왔다. 이 연구의 목적은 간에서만 Niemann-Pick C1-Like 1 (NPC1L1)이 발현된 생쥐모델(L1LivOnly) -담즙으로 스테롤 분비와 장에서의 스테롤 흡수가 모두 결핍된 동물 모델-에서 달수의 콜레스테롤 역수송에 어떤 역할을 하는지와 NPC1L1 억제제인 ezetimibe가 간의 NPC1L1을 억제함으로써 대식구의 콜레스테롤 역수송을 촉진하는지를 알아보는 것이다.

방법 및 결과
L1LivOnly 생쥐는 NPC1L1 결핍 생쥐(L1-KO)와 간에 서 사람 NPC1L1을 과발현시킨 생쥐를 교배를 통해 얻었다. 대식구 대 대변 콜레스테롤 역수송은 L1-KO과 L1LivOnly 생쥐의 복장으로 C57BL/6 생쥐에 서 얻은 [3H]-콜레스테롤을 스테롤 분비 억제와 대식구 대 대변 콜레스테롤 역수송 조절에 어떤 역할을 하는지와 NPC1L1 억제제인 ezetimibe가 간의 NPC1L1을 억제함으로써 대식구의 콜레스테롤 역수송을 촉진하는지를 알아보는 것이다.

결론
장에서의 콜레스테롤 흡수가 결핍된 생쥐에서 대식구 대 대변으로의 콜레스테롤 역수송은 담즙으로의 스테롤 분비의 효율에 의해 결정되며 ezetimibe는 간의 NPC1L1 기능을 억제함으로써 대식구의 콜레스테롤 역수송을 촉진한다.
클레스테롤 역수송(reverse cholesterol transport, RCT)이란 일반적으로 말초 조직의 잉여 클레스테롤을 HDL에 의해 간으로 전달하는 과정을 말한다. 이후 간으로 전달된 클레스테롤은 담즙으로 분비되어 결국 대변으로 빠져나감로 2010년 Temel 등의 연구에서는 수술을 통해 소장으로의 담즙 배출을 막더라도 정상적으로 macrophage RCT가 유지될을 보여 주어 TICE의 근거를 더하였다. TICE는 LXR 작용제, PPARδ, 고지방식이, 식물 sterol에 의해 증가하는 것으로 알려져 있으나, 실제 작용기전이 무엇인지 즉, 매개하는 단백 혹은 수용체가 무엇인지 아직 밝혀지지 않아 많은 연구가 필요한 분야이다. 동물실험에 의하면 TICE에 의한 콜레스테롤 배출이 30~40% 정도를 차지하며, LXR 작용제 처리시 hepatobiliary excretion에 비해 TICE가 훨씬 많이 증가하며, 담즙으로의 과다한 클레스테롤 분비시 걱정되는 담석 형성 등의 문제도 없기 때문에 향후 약물 개발의 좋은 표적이 될 것으로 생각된다.

이 연구에서 사용한 ezetimibe는 NPC1L1 억제제인데, NPC1L1은 생쥐에서는 소장에만 분포하나 사람에서는 간과 소장에 모두 분포하고 있으므로 사람에서는 클레스테롤 대사에서 hepatic NPC1L1의 역할이 있을 것으로 생각된다. 담즙으로의 클레스테롤 분비가 감소하면 장에서의 콜레스테롤 흡수 속도가 감소하고 콜레스테롤 합성이 증가하기 때문에 macrophage RCT를 생각할 때는 서로 연관되어 있는 담즙으로의 클레스테롤 분비와 장에서의 클레스테롤 흡수 속도 변화의 영향을 함께 고려해야 한다. 그러나 본 연구에서 사용된 간에서의 콜레스테롤 농도 감소시에 macrophage RCT의 증가로 인한 담즙으로의 클레스테롤 분비 감소는 장에서의 클레스테롤 흡수 속도 변화를 보조적으로 향상시킬 수 있다. 하지만 실제 생체에서는 이 두 단계가 개별적으로 작용하므로 이 연구의 가치가 기존 Temel 등의 연구보다 더 의미 있다고 할 수 있는지에 대해서는 의구심이 있다. NPC1L1 저해제인 ezetimibe 투여시 소장에서
콜레스테롤 흡수가 감소하여 macrophage RCT 가 증가한다고 생각해왔으나 이 연구에서 보면 간의 NPC1L1 억제를 통해 biliary cholesterol secretion의 증가를 유도하여 macrophage RCT 가 증가함을 보여준 것은 매우 흥미롭다. 논문 에 언급된 대로 사람에서 ezetimibe가 콜레스 테롤 역수송에 미치는 영향을 보는 ezetimibe reverse cholesterol transport (RCT) pilot study 의 결과가 기대된다. Clinicaltrials.gov에 제시 된 preliminary result에 의하면, 이 연구에는 ezetimibe군 26명, placebo군 26명이 등록되어 7주간 치료 후 혈장 유래 콜레스테롤의 fecal excretion을 1차 지표로 살펴보았다. 결과는 기대했던 대로 ezetimibe군에서 혈장 유래 콜레스테롤의 fecal excretion이 유의하게 높았다. 그러나 이로 인한 macrophage RCT의 차이는 아직 분석되지 못하였다. 향후 발표된 이 연구의 결과가 기대된다.

REFERENCES