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

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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.

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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.1,2 This process is believed to explain, at least in part, why increased plasma HDL-cholesterol is atheroprotective.3 Recently, an intestinal route for mass cholesterol excretion in the feces has been reported,4,5 which has promoted studies of the significance of this nonbiliary route in RCT, using genetic and surgical mouse models deficient in biliary cholesterol secretion.6,7

One genetic model used in macrophage RCT assays is the transgenic mice specifically overexpressing human Niemann-Pick C1-Like 1 (NPC1L1) in liver.6,8 Unlike mice lacking the ATP-binding cassette (ABC) transporter B4 (ABC4), which are deficient in both biliary cholesterol/phospholipids secretion and liver cholesterol,9 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.10 The function of NPC1L1 can be inhibited by ezetimibe,10,11 a potent cholesterol absorption inhibitor, developed to lower blood cholesterol.12 In humans, NPC1L1 is expressed in liver in addition to intestine.13,14,15 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.3

Using in vivo RCT assay protocol of Rader,16 Temel et al17 showed that biliary sterol secretion is not required for macrophage RCT in NPC1L1 liver transgenic mice, and in...
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\textsuperscript{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, ~50% of cholesterol in the gut lumen is absorbed in humans and rodents,\textsuperscript{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,\textsuperscript{19,20} a model that does not express NPC1L1 in liver.\textsuperscript{19} Altered biliary cholesterol secretion was reported to influence intestinal cholesterol absorption rates.\textsuperscript{21,22} Acute biliary diversion or bile duct ligation reduces intestinal cholesterol absorption and profoundly alters intestinal metabolism, including increases in intestinal cholesterol synthesis.\textsuperscript{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\textsuperscript{10} to liver-specific NPC1L1 transgenic mice\textsuperscript{a} and generated mice expressing no endogenous NPC1L1, but human NPC1L1 in liver only (L1LivOnly mice).\textsuperscript{10} We have previously shown that ezetimibe treatment increases biliary sterol excretion by inhibiting hepatic NPC1L1.\textsuperscript{8,25} This observation raised an interest-

Figure 1. Ezetimibe treatment restores biliary cholesterol output in L1 LivOnly mice. Lipid concentrations and molar ratios in the gallbladder bile of L1-KO mice (n=5), L1 LivOnly mice (n=6), and L1 LivOnly&Eze mice treated with ezetimibe (L1 LivOnly&Eze; n=4). A, Biliary cholesterol. B, Biliary phospholipids. C, Biliary bile acids. D, Fecal neutral sterol excretion in L1-KO (n=8), L1 LivOnly (n=9), and L1 LivOnly&Eze (n=7) mice. *P<0.05 between a and b groups for each measurement (ANOVA)."
Figure 3. 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.

(from 30.92 µmol/d per 100 g BW in L1LivOnly group to 45.17 µmol/d per 100 g BW in L1LivOnly&Eze group; Figure 1D), suggesting a substantial contribution of biliary cholesterol secretion to fecal cholesterol excretion in our animals on the low-cholesterol diet (0.015%).

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.

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

To measure macrophage RCT, we first determined how hepatic NPC1L1 overexpression influences homeostasis and distribution of [3H]-cholesterol derived from intra-peritoneally 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 were measured during in vivo macrophage RCT studies. Consistent with elevated plasma cholesterol in L1LivOnly mice, plasma [3H]-tracer levels were significantly higher in L1LivOnly compared with L1-KO mice at 6, 24, and 48 hours after peritoneal injection of [3H]-cholesterol–labeled primary macrophages (Figure 3A). [3H]-tracer contents were also increased in several tissues of L1LivOnly mice, including liver, small intestine, lung, and spleen (Figure 3B). In this RCT experiment, we included a group of L1-KO mice treated with ezetimibe to determine whether ezetimibe alters plasma and tissue levels of [3H]-tracer during macrophage RCT assays when NPC1L1 is disrupted. Consistent with NPC1L1 being the target of ezetimibe,12,13 ezetimibe treatment had no effects on plasma and tissue [3H]-tracer levels in L1-KO mice (Figure 3).

**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).

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 ≈60% (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
reduced in L1 LivOnly mice (Figure 5), suggesting that reduced hepatic overexpression of NPC1L1 affects HDL
content in blood and tissues and restored biliary and fecal excretion of [3H]-neutral sterols in L1 LivOnly mice. Our results demonstrated 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, 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. 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.

Although 1 study performed by Temel et al 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 al 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 al that “biliary cholesterol secretion represents the major pathway relevant for RCT” in mice, but in contrast with the finding by Temel et al. 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

**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 disposal. To determine whether hepatic overexpression of NPC1L1 affects HDL turnover and tissue uptake, we injected [3H]-Cholesteryl Oleyl Ether ([3H]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 the injection. Despite a significant reduction in the fractional catabolic rate, hepatic and intestinal uptake of [3H]CEt-HDL was not reduced in L1 LivOnly mice (Figure 5), suggesting that reduced macrophage-to-feces cholesterol transport in L1 LivOnly mice may not be a result of decreased availability of liver cholesterol for biliary disposal.

**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 (L1 LivOnly) 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 L1 LivOnly mice. Our results demonstrated an essential role of biliary sterol secretion in mediating macrophage-to-feces RCT in mice deficient in intestinal cholesterol absorption.
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 demonstrated that biliary cholesterol secretion is essential for macrophage 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 macrophage 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−/− mice. Consistently, the present work showed that hepatic overexpression of human NPC1L1 inhibits 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−/− 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 macrophage-to-feces RCT by inhibiting hepatic NPC1L1. It has been previously reported by others that ezetimibe treatment stimulates macrophage RCT in wild-type mice. In those studies, the effect of ezetimibe on RCT should be considered as a result of inhibition of intestinal cholesterol absorption because mice do not normally express measurable NPC1L1 in the liver. However, the livers of humans and monkeys express NPC1L1.

A clinical trial [NCT00701727, Ezetimibe RCT Pilot Study. Weblink: http://clinicaltrials.gov/show/NCT00701727] was initiated to examine the effect of ezetimibe on RCT in humans. On the basis of the results posted on the Web site of this trial, ezetimibe treatment at 10 mg/d for 7 weeks significantly increased fecal excretion of plasma-derived cholesterol in hypercholesterolemic humans. Our present work suggests that ezetimibe may promote RCT in humans through inhibiting NPC1L1 function in both intestine and liver. In conclusion, biliary sterol secretion plays a key role in promoting macrophage-to-feces RCT. Ezetimibe promotes macrophage 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 macrophage RCT.

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Disclosures

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### Significance

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.
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Materials and Methods

Mice and Diets

$\text{L1}^{\text{LivOnly}}$ mice were generated by crossing $\text{L1-KO}$ mice with liver-specific NPC1L1 transgenic mice. The genetic background of $\text{L1}^{\text{LivOnly}}$ 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) 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 $\text{L1-KO}$ and $\text{L1}^{\text{LivOnly}}$ 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. After being fed the diet for 14 days, a subgroup of $\text{L1}^{\text{LivOnly}}$ mice were switched to the same diet supplemented with 0.005% (w/w) of ezetimibe.

In Vivo Macrophage RCT Studies

The in vivo macrophage-to-feces RCT assay was performed according to the protocol developed by Rader and colleagues. 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 [$^3$H]-cholesterol for 24 h in 10% FBS-
containing DMEM medium supplemented with a mixture of 5 μCi/ml [\(^3\)H]-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. [\(^3\)H]-cholesterol labeling efficiency was measured by extracting lipids from an aliquot of cells using the method of Bligh and Dyer\(^5\) 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 (~3x10^6 cells/ml at ~2x10^6 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.

\[^3\text{H}]-\text{Tracer Measurements in Bile, Feces, and Tissues}\n
Lipids in ~10 μl of gallbladder bile from each mouse were extracted by sequentially adding/vortexing 1 ml H\(_2\)O, 3 ml methanol:chloroform (2:1), 2 ml chloroform, and 1 ml H\(_2\)O 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 [\(^3\)H]-bile acids, and the lower organic phase contained [\(^3\)H]-cholesterol. A known volume of each phase was dried under N\(_2\) and resuspended in 5 ml scintillation cocktail (Bio-Safe II,
Order#:111195, Research Product International Corp., Mount Prospect, IL) for the
expressed as the percentage of $[^3]$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
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

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 $[^3]$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. For analysis of hepatic lipid contents, the lipids were extracted from ~80 mg of liver tissues and measured enzymatically as described previously. Biliary concentrations of free cholesterol, phospholipids and bile acids were determined as described previously.

Measurements of Fecal Neutral Sterol Excretion

The mice were individually housed. The feces were collected for 48 h and dried in a 70°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 µg of 5α-cholestane as an internal standard. The feces were saponified in 2 ml of 95% ethanol and 200 µl of 50% KOH (w/v in water) on a 65°C heating block for 2 h. The lipids were extracted by adding 2 ml hexane and 2 ml H₂O. 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 [³H]cholesteryl oleyl ether exactly as described previously. [³H]CEt-HDL was dialyzed with PBS and radioactivity was then counted. [³H]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 [$^3$H]CEt-HDL injection as described by Nijstas E, et al. The fractional catabolic rate (FCR) was calculated from the decay curves according to the Matthews method. The organ uptake of [$^3$H]CEt-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).

**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.

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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)이 반영된 생쥐모델(L1LivOnly)-담즙으로 스테롤 분비와 장에서의 스테롤 흡수가 모두 결핍된 동물모델에서 담즙 콜레스테롤 분비가 대식구의 콜레스테롤 역수송 조절에 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 간에서 사람 NPC1L1을 과발현시킨 생쥐를 교배하여 얻었다. 대식구 대 대변 콜레스테롤 역수송은 L1-KO와 L1LivOnly 생쥐의 복강으로 C57BL/6 생쥐에서 얻은 [3H]-콜레스테롤로 표시된 복강내 대식구를 주입하여 평가하였다. 간에서 NPC1L1 과발현에 의한 담즙 스테롤 분비 억제는 L1LivOnly 생쥐에서 담즙 분획으로, 추적자(tracer) 배출에는 영향을 미치지 않으면서 1차 복강내 대식구에서 담즙과 대 변의 중립적 스테롤 분획(neutral sterol fraction)으로의 [3H]-콜레스테롤 수송을 뚜렷이 감소시켰다. 2주간의 ezetimibe 치료는 L1LivOnly 생쥐의 중립적 스테롤 분획에서 담즙과 대변의 [3H]-추적자 배설을 완전히 회복시켰다. 고밀도지단백 동력학 연구에서 L1LivOnly 생쥐는 L1-KO 생쥐와 비교하여 간과 장에서 고밀도지단백 콜레스테롤 에테르(high-density lipoprotein–cholesterol ether)의 흡수는 차이가 없었으나 분해 속도(fractional catabolic rate)는 유의하게 감소되었다.

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.
 Commentary

콜레스테롤 역수송(reverse cholesterol transport, RCT)이란 일반적으로 말초 조직의 잉여 콜레스테롤을 HDL에 의해 간으로 전달하는 과정을 말한다. 이후 간으로 전달된 콜레스테롤은 담즙으로 분비되어 결국 대변으로 빠져나간다. 이는 40여년 전 Glomset 등이 소개한 개념으로 높은 HDL 혈중 농도는 RCT를 증가시키고 결국 죽상경화증 발생을 억제하는 효과가 있다. 그러나 ABCA1 결핍 생쥐는 HDL가 전혀 없지만 biliary 또는 fecal cholesterol excretion이 정상이다. 그리고 HDL이 결핍된 apoA-I-/- mice에 rHDL를 주더라도 fecal sterol excretion이 증가되지 않는다. 또한, LCAT heterozygous mice는 심한 휴달 HDL의 감소가 있지만 대식구에서 분변으로의 RCT는 유지된다는 결과를 보인 동물 실험에서 보면 HDL이 RCT의 유일한 경로가 아닌 점을 시사한다. 2007년 van der Velde 등은 이러한 전통적인 hepatobiliary cholesterol secretion 외에 장에서 직접 혈액 속의 콜레스테롤을 제거하는 기전이 있을음을 주장하면서 transintestinal cholesterol excretion (TICE)이라는 개념을 주장하였다. TICE의 근거로는 1) Multidrug resistance 2 (Mdr2) 결핍 생쥐의 경우 인지질을 담즙으로 분비할 수 없어 biliary cholesterol level이 80% 이상 감소되어 있지만 fecal cholesterol excretion은 정상이거나 증가되어 있다. 2) Mdr2 결핍 생쥐에 liver X receptor (LXR)를 활성화시키면 biliary cholesterol output의 증가 없이 fecal sterol output이 크게 증가한다. 3) 간에서 NPC1L1은 과발현시키면 biliary cholesterol levels이 90% 이상 감소하지만, fecal neutral sterol loss와 macrophage RCT는 정상으로 유지된다. 나이가 2010년 Temel 등의 연구에서는 수술을 통해 소장으로의 담즙 배출을 막더라도 정상적으로 macrophage RCT가 유지될을 보여 주어 TICE의 근거를 다하였다. TICE는 LXR 작용제, PPARδ, 고지방식이, 식물 sterol에 의해 증가하는 것으로 알려져 있지만, 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 콜레스테롤 분비의 문제가 없기 때문에 향후 약물 개발의 좋은 표적이 될 것으로 생각된다.

이 연구에서 사용한 ezetimibe는 NPC1L1 억제제인데, NPC1L1은 생쥐에서는 소장에만 분포하지만 사람에서는 간과 소장에 모두 분포하고 있으므로 사람에서는 콜레스테롤을 대사에서 hepatic NPC1L1의 역할이 있을 것으로 생각된다. 담즙으로의 콜레스테롤 분비가 감소하면 장에서의 콜레스테롤 흡수 속도가 감소하고 콜레스테롤 합성이 증가하기 때문에 macrophage RCT를 생각할 때는 서로 연관되어 있는 담즙으로의 콜레스테롤 분비와 장에서의 콜레스테롤 흡수 속도 변화의 영향을 함께 고려해야 한다. 그런 면에서 본 연구에서 사용된 간에서만 NPC1L1이 과발현된 NPC1L1 결핍 생쥐모델의 가치가 있다고 할 수 있다. 하지만 실제로 생체, 특히 사람에서는 이 둘의 인자가 함께 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 RCT가 증가한다고 생각해왔으나 이 연구에서 보면 간의 NPC1L1 억제를 통해 biliary cholesterol secretion의 증가를 유도하여 macrophage RCT 가 증가함을 보여준 것은 매우 흥미롭다. 논문에 언급된대로 사람에서 ezetimibe가 콜레스테롤 역수송에 미치는 영향을 보는 ezetimibe reverse cholesterol transport (RCT) pilot study의 결과가 기대된다. Clinicaltrials.gov에 제시된 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 발표될 이 연구의 결과가 기대된다.

REFERENCES