Niemann-Pick C1-Like 1 Is Required for an LXR Agonist to Raise Plasma HDL Cholesterol in Mice

Weiqing Tang, Yinyan Ma, Lin Jia, Yiannis A. Ioannou, Joanna P. Davies, Liqing Yu

Objectives—Activation of liver x receptor (LXR) raises plasma HDL-cholesterol (HDL-C) in mice. Interestingly, the LXR agonist GW3965 fails to raise plasma HDL-C in mice lacking intestinal ABCA1, indicating that intestinal ABCA1 plays a predominant role in GW3965-mediated HDL production. How this is coupled to intestinal function remains elusive. Because cholesterol is essential for HDL assembly and directly regulates intestinal ABCA1 expression via activating LXR, we hypothesized that cholesterol absorption, a major function of intestine, modulates LXR-dependent HDL formation.

Methods and Results—Mice lacking Niemann-Pick C1-Like 1 (NPC1L1) (L1-KO mice), a gene that is essential for cholesterol absorption, were treated with LXR agonist T0901317 for 7 days. Intriguingly, this treatment failed to significantly raise plasma HDL-C but caused a much greater fecal cholesterol excretion in L1-KO mice. The intestinal ABCA1 mRNA level was about 4-fold lower in L1-KO versus wild-type mice, and increased 3.9-fold and 8.8-fold after T0901317 treatment in wild-type and L1-KO mice, respectively. Hepatic ABCA1 failed to respond to T0901317 in mice of both genotypes, although hepatic mRNAs for many LXR target genes were higher in the T0901317-treated versus untreated wild-type animals.

Conclusions—NPC1L1 is required for an LXR agonist to increase plasma HDL-C in mice. (Arterioscler Thromb Vasc Biol. 2008;28:000-000.)

Key Words: cholesterol absorption ■ Niemann-Pick C1-Like 1 ■ liver x receptor ■ HDL ■ ABCA1

Plasma high density lipoprotein (HDL) is protective against coronary heart disease. It is believed that HDL protects the cardiovascular system by transporting excess cholesterol from peripheral tissues back to the liver for clearance, a process that is known as reverse cholesterol transport. The biogenesis of plasma HDL requires a functional ATP-binding cassette (ABC) transporter, ABCA1, which mediates assembly of free cholesterol and phospholipids with apolipoprotein A-I (apoA-I) to form nascent HDL particles. ABCA1 is expressed in many tissues, including the liver, intestine, and macrophages. Bone marrow transplantation studies have shown that the macrophage does not play an important role in the formation of plasma HDL under physiological conditions. Liver-specific ablation of ABCA1 in mice has established liver as the major source of HDL. Interestingly, mice lacking ABCA1 specifically in intestine showed a 30% reduction in plasma HDL, indicating that intestinal ABCA1 directly contributes to HDL biogenesis.

ABCA1 is a target gene of the nuclear receptor liver x receptor (LXR). Oral administration of synthetic LXR agonists (T0901317 or GW3965) increases plasma HDL-cholesterol (HDL-C) levels in wild-type mice, and this effect is abolished in whole-body ABCA1-null mice. It remains unclear which tissue is more important in LXR agonist-induced increases in plasma HDL-C. GW3965, a compound that specifically activates intestinal LXR target genes without much effect on hepatic LXR targets, can raise plasma HDL-C in the liver-specific but not intestine-specific ABCA1 knockout mice, suggesting that intestinal ABCA1 may contribute to the increased plasma HDL-C induced by LXR activation.

How the LXR agonist-induced and intestinal ABCA1-dependent increase in plasma HDL-C is coupled to intestinal function remained to be elucidated. Evolved as an absorptive organ, the small intestine functions primarily to absorb various nutrients, including cholesterol. In the intestinal lumen, cholesterol comes from 2 sources: diet and bile. Normally, about 50% of cholesterol in the gut lumen is absorbed, which has an evolutionary advantage because de novo cholesterol biosynthesis requires tremendous amounts of energy input. Absorbed free cholesterol can be esterified by acyl-coenzyme A (CoA) acyltransferase 2 (ACAT2) to form cholesterol esters for incorporation into chylomicrons, or can interact with ABCA1 for assembly into nascent HDL particles, although it was unknown how important the absorbed free cholesterol is in regulating intestinal HDL formation.
Niemann-Pick C1-Like 1 (NPC1L1) was recently identified to be required for cholesterol absorption. Mice lacking NPC1L1 (L1-KO mice) have a substantial reduction in cholesterol absorption, and are completely resistant to diet-induced hypercholesterolemia and to apoE deficiency-caused atherosclerosis. The expression level of intestinal cholesterol absorption directly modulates intestinal ABCA1 expression levels.

Based on the aforementioned facts, we hypothesized that intestinal cholesterol absorption may play an important role in LXR agonist-stimulated HDL formation. To test this hypothesis, L1-KO mice were treated with T0901317, and their lipids in plasma and tissues were analyzed. Consistent with our hypothesis, T0901317 failed to increase plasma HDL-C in L1-KO mice. Thus, intestinal cholesterol absorption is required for T0901317 to raise plasma HDL-C in mice.

**Methods**

Wild-type (WT) and L1-KO mice (n=6) were treated with either vehicle (V) or T0901317 (T). The concentrations of plasma total cholesterol (TC), free cholesterol (FC), phospholipids (PL), and triglyceride (TG) were analyzed. Cholesterol ester concentration was calculated by multiplying the difference between total and free cholesterol mass by 1.67. The differences between values associated with different superscript letters in each parameter is statistically significant (P<0.05).

**Results**

**Table 1. Plasma Lipid Concentrations**

<table>
<thead>
<tr>
<th>Genotype</th>
<th>TC Mean±SEM (mg/dl)</th>
<th>FC Mean±SEM (mg/dl)</th>
<th>CE Mean±SEM (mg/dl)</th>
<th>PL Mean±SEM (mg/dl)</th>
<th>TG Mean±SEM (mg/dl)</th>
</tr>
</thead>
<tbody>
<tr>
<td>WT-V</td>
<td>142.5±14.0&lt;sup&gt;a&lt;/sup&gt;</td>
<td>30.8±1.2&lt;sup&gt;a&lt;/sup&gt;</td>
<td>186.8±9.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>267.2±10.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.0±7.4&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>WT-T</td>
<td>270.5±21.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>82.7±7.5&lt;sup&gt;b&lt;/sup&gt;</td>
<td>313.7±25.2&lt;sup&gt;b&lt;/sup&gt;</td>
<td>464.0±17.3&lt;sup&gt;b&lt;/sup&gt;</td>
<td>41.7±7.5&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>L1-KO-V</td>
<td>105.5±6.9&lt;sup&gt;a&lt;/sup&gt;</td>
<td>23.5±2.5&lt;sup&gt;a&lt;/sup&gt;</td>
<td>136.9±9.3&lt;sup&gt;a&lt;/sup&gt;</td>
<td>228.0±8.6&lt;sup&gt;a&lt;/sup&gt;</td>
<td>48.0±8.6&lt;sup&gt;a&lt;/sup&gt;</td>
</tr>
<tr>
<td>L1-KO-T</td>
<td>132.2±12.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>34.2±2.7&lt;sup&gt;a&lt;/sup&gt;</td>
<td>163.7±17.1&lt;sup&gt;b&lt;/sup&gt;</td>
<td>251.8±25.8&lt;sup&gt;b&lt;/sup&gt;</td>
<td>30.8±2.6&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

Wild-type (WT) and L1-KO mice (n=6) were treated with either vehicle (V) or T0901317 (T). The concentrations of plasma total cholesterol (TC), free cholesterol (FC), phospholipids (PL), and triglyceride (TG) were analyzed. Cholesterol ester concentration was calculated by multiplying the difference between total and free cholesterol mass by 1.67. The differences between values associated with different superscript letters in each parameter is statistically significant (P<0.05).

**Figure 1.** A, Lipoprotein cholesterol distribution in pooled plasma from wild-type (WT) mice (n=6) treated with either vehicle (V) or T0901317 (T). B, Lipoprotein cholesterol distribution in pooled plasma from L1-KO mice (n=6) treated with either vehicle or T0901317. This experiment was repeated with a different set of animals and identical results were obtained. C, Western blot of plasma apoE mass in 3 samples from each group.

**Statistical Analysis**

All data are represented as the mean±SEM. Statistically significant differences were determined by ANOVA (Tukey-Kramer honestly significant difference) for all values except qPCR data that were statistically analyzed by unpaired, 2-tailed Student t test. P<0.05 was considered statistically significant.

**LXR Agonist Treatment**

At 2 months of age, male wild-type and L1-KO mice of C57BL/6 background were fed a synthetic diet containing 10% energy from palm oil and 0.015% (w/w) cholesterol (prepared at the institutional diet core) until mice were euthanized. From day 15 of diet feeding, mice were gavaged daily for 7 days with either a 100 mg/kg of T0901317 (Cayman Chemical Company) dissolved in vehicle solution (dimethyl sulfoxide/phosphate-buffered saline [1:3]), or 25 mg/kg of T0901317 (Cayman Chemical Company) dissolved in vehicle solution. All mice were then either euthanized for tissue and blood collection after a 4-hour fast or used for bile duct cannulation.

**Discussion**

To directly test our hypothesis that intestinal cholesterol absorption is required for an LXR agonist to increase plasma HDL-C, plasma concentrations of lipids and lipoprotein cholesterol distribution were determined. As expected, in wild-type mice, T0901317 treatment raised plasma concentrations of total cholesterol, free cholesterol, cholesterol ester, and phospholipids (Table 1), and the increased cholesterol was accumulated in large HDL particles (Figure 1A). Interestingly, all of these changes were either abolished or attenuated in L1-KO mice (Table 1 and Figure 1B). Plasma triglyceride concentrations failed to increase in response to T0901317 treatment in mice of both genotypes (Table 1).
The attenuated increase in plasma lipoprotein cholesterol in response to T0901317 was also seen in L1-KO versus wild-type mice when T0901317 was administered by intraperitoneal injection (supplemental Figure 1), indicating that the compound availability was not responsible for the observed attenuation. It was noticed that the increase in plasma cholesterol in the T0901317-treated chow-fed wild-type mice was not as high as that in the T0901317-treated wild-type mice fed the synthetic diet (WT-T in supplemental Figure I versus WT-T in Figure 1A). This might be attributable to differences in diet or the administration route of T0901317. Alternatively, to achieve maximal effect of T0901317 on plasma cholesterol, intestine might have to be the first organ being exposed to T0901317. If this is the case, it would further support that intestine plays a crucial role in LXR activation-induced increase in plasma cholesterol.

It has been demonstrated that large HDL particles accumulated in the T0901317-treated animals are apoE-enriched. Consistently, T0901317 increased plasma apoE mass in wild-type mice, but this increase was modest in L1-KO mice (Figure 1C). Compared with vehicle-treated wild-type mice, a slight reduction in plasma apoE mass was also observed in vehicle-treated L1-KO mice.

T0901317 Increases Intestinal but not Hepatic ABCA1 Expression

Because ABCA1 directly controls plasma HDL-C levels, hepatic ABCA1 expression was examined. Under our experimental conditions, T0901317 failed to increase ABCA1 mRNA and protein levels in the liver of both wild-type and L1-KO mice (Figure 2), which was gene-specific because the hepatic levels of ABCG5 and ABCG8, the 2 target genes of LXR, were increased in response to T0901317 in wild-type mice, despite that the increase did not reach statistical significance (Figure 2).

The expression levels of other genes relating to lipoprotein metabolism were also examined. After T0901317 treatment, the hepatic protein level of low density lipoprotein (LDL) receptor (LDLR) was increased in wild-type mice but not in L1-KO mice (Figure 2B). The LDLR mRNA levels were slightly increased in T0901317-treated mice of both genotypes and in L1-KO versus wild-type mice. However, these increases were not statistically significant (Figure 2A). Interestingly, the changes of hepatic mRNAs for proprotein convertase subtilisin/kexin type 9, a protein that mediates LDLR degradation, not only mirrored LDLR mRNA alterations, but also were highly statistically significant among all groups (Figure 2A). The hepatic protein levels of the HDL receptor SR-BI were reduced in T0901317-treated mice of both genotypes (Figure 2B). The hepatic SR-BI mRNA levels were significantly reduced accordingly (Figure 2A). No changes were seen in hepatic apoE mRNA levels among all groups, in spite of an increase in plasma apoE mass in T0901317-treated mice (Figure 2B).

In contrast to hepatic ABCA1 expression, after T0901317 treatment, intestinal ABCA1 mRNA levels significantly increased 3.9-fold and 8.8-fold in wild-type and L1-KO mice, respectively (Figure 2C). Because the basal level of intestinal ABCA1 mRNA was reduced by 75% in L1-KO versus mice, the net abundance of intestinal ABCA1 mRNA was still 42% lower in L1-KO mice than in wild-type mice after T0901317 treatment (Figure 2C). The pattern of ABCG5 and ABCG8 mRNA variations was similar to ABCA1, but the pattern of statistical significances was slightly different (Figure 2C).
LXR Activation by T0901317 Causes a Greater Fecal Neutral Sterol Excretion in L1-KO Mice

To further probe how disruption of NPC1L1 renders mice resistant to T0901317-induced increases in plasma HDL-C, the cholesterol balance was assayed by measuring fecal excretion of neutral sterols (cholesterol and its bacterial modified metabolites). As expected, T0901317-treated L1-KO mice had the highest fecal neutral sterol excretion rate, followed by L1-KO mice on vehicle, wild-type mice on T0901317, and wild-type mice on vehicle (Figure 3A). Interestingly, the majority of cholesterol was converted to coprostanol, a bacterial metabolite of cholesterol, in T0901317-treated L1-KO mice with the coprostanol to cholesterol ratio as high as 4.2, whereas these ratios ranged from 0.3 to 0.5 in all other groups (Figure 3B).

Surprisingly, a reduction in stool output was observed for the first time in T901317-treated mice on our synthetic diet (Figure 3C). Interestingly, compared with wild-type mice, L1-KO mice also showed a reduction in stool output, regardless of treatments. This resulted in a \( \approx 50\% \) decrease in stool output in T0901317-treated L1-KO mice versus vehicle-treated wild-type mice.

T0901317 Increases Biliary Cholesterol Concentrations in Both Wild-Type and L1-KO Mice

It has been established that T0901317 promotes biliary cholesterol excretion, and this effect is dependent on the heterodimeric cholesterol exporter ABCG5/ABCG8. To examine whether NPC1L1 disruption or block of intestinal cholesterol absorption alters T0901317-induced hepatobiliary cholesterol hypersecretion, the concentrations of biliary lipids were determined in 2 consecutive 15-minute bile samples collected from the common bile duct. As shown in Table 2, T0901317 administration significantly augmented biliary cholesterol concentrations in both bile samples from mice of both genotypes. This effect was cholesterol-specific because the concentrations of phospholipids and bile acids were largely unaffected. Increased biliary cholesterol concentrations was likely a result of increased expression of ABCG5 and ABCG8 (Figure 2A and 2B), which would be consistent with ABCG5/ABCG8 being the direct target of LXR and the major hepatobiliary cholesterol transporter.

Disruption of NPC1L1 Attenuates T0901317-Induced Hepatic Steatosis

LXR activation by T0901317 causes overaccumulation of triglyceride in the liver as a result of increased lipogenesis. In this study, T0901317 treatment for 7 days caused a 6.7-fold increase in hepatic triglyceride content in wild-type mice, but only a 2.3-fold increase in L1-KO mice (Figure 4A). No changes in the hepatic content of total cholesterol, free cholesterol, cholesterol ester, and phospholipids were observed.
observed (data not shown). Attenuation of hepatic triglyceride accumulation in T0901317-treated L1-KO mice was coupled to a reduced increase in the liver/body weight ratios (Figure 4B) and in the hepatic mRNAs for lipogenic genes including sterol regulatory element-binding protein-1c (SREBP-1c), fatty acid synthase (FAS), and stearoyl-CoA desaturase-1 (SCD-1) (Figure 4C).

**Discussion**

The major finding in this study is that disruption of NPC1L1 attenuated the T0901317-induced increase in plasma HDL-C in mice. Because NPC1L1 is almost exclusively expressed in intestine in mice, and ablation of NPC1L1 causes a defect in intestinal absorption of sterols, this finding suggests that intestinal cholesterol absorption is required for T0901317 to increase plasma HDL-C in mice. It also indicates that the intestine is the major source of increased HDL-C seen in T0901317-treated mice.

How does intestinal cholesterol absorption influence LXR agonist-induced increase in plasma HDL-C? A previous study has shown that intestinal but not hepatic ablation of ABCA1 abolished LXR agonist GW3965-stimulated elevation in plasma HDL-C, indicating a critical role of intestinal ABCA1 in mediating this LXR effect. Additionally, hepatic ABCA1 expression has been shown to be insensitive to LXR activation either by cholesterol feeding or synthetic LXR agonists, which is consistent with T0901317 failing to increase hepatic ABCA1 expression in both wild-type and L1-KO mice (Figure 2).

One reasonable explanation for T0901317 failing to induce an increase in plasma HDL-C in the absence of cholesterol absorption could be that the amount of cholesterol absorbed from the gut lumen directly determines the baseline level of ABCA1 via modulating LXR-dependent ABCA1 expression. In the case of impaired cholesterol entry into enterocytes from the gut lumen as seen in L1-KO mice, the baseline level of ABCA1 is significantly downregulated because there is less cholesterol to activate LXR. Although T0901317 can directly activate LXR and upregulate ABCA1 expression in the intestine of L1-KO mice (8.8-fold upregulation in L1-KO mice versus 3.9-fold upregulation in wild-type mice), the absolute level of ABCA1 is still 42% lower than that in T0901317-treated wild-type mice (Figure 2C), which may not be high enough to significantly raise plasma HDL-C. It is also highly possible that the block in cholesterol absorption leads to less cholesterol available for ABCA1-dependent lipidation in the intestine, thereby reducing HDL-C formation.

Alternatively, despite that T0901317 significantly reduced the SR-BI expression in both genotypes (Figure 2A and 2B), a role of hepatic SR-BI could not be excluded because the hepatic mRNA and protein levels of SR-BI were still higher in L1-KO versus wild-type mice after T0901317 treatment, and the relative higher hepatic level of SR-BI may result in more plasma HDL-C particles being cleared in L1-KO mice. To the best of our knowledge, this study is the first to show that T0901317 reduces hepatic SR-BI expression. How T0901317 regulates hepatic SR-BI protein remains to be elucidated.

ApoE has been shown to be enriched in, and required for T0901317 to raise large HDL particles. Because T0901317 had no effect on hepatic apoE mRNA (Figure 2A), the difference in plasma apoE mass between T0901317-treated wild-type and L1-KO mice after T0901317 treatment, and the relative higher hepatic level of SR-BI may result in more plasma HDL-C particles being cleared in L1-KO mice. To the best of our knowledge, this study is the first to show that T0901317 reduces hepatic SR-BI expression. How T0901317 regulates hepatic SR-BI protein remains to be elucidated.

ApoE has been shown to be enriched in, and required for T0901317 to raise large HDL particles. Because T0901317 had no effect on hepatic apoE mRNA (Figure 2A), the difference in plasma apoE mass between T0901317-treated wild-type and L1-KO mice might be attributed to extrahepatic modulation of apoE, such as plasma apoE turnover and extrahepatic synthesis. ApoE is a target of LXR in macrophages and adipocytes. It is possible that T0901317 failed to fully induce apoE expression in these cells as seen in livers for some lipogenic genes (Figure 4C) when intestinal cholesterol absorption is blocked, thereby causing reduced secretion.
of apoE into plasma in L1-KO mice. If this is the case, failure of T0901317 to raise plasma HDL-C may partially be attributed to altered apoE metabolism.

An interesting finding from this study is that LXR activation by T0901317 causes less hepatic accumulation of triglyceride and reduced activation of lipogenic genes in L1-KO mice than in wild-type mice (Figure 4). L1-KO mice have a deficit in cholesterol balance as a result of defect in intestinal absorption of both dietary and biliary cholesterol. After T0901317 treatment, this deficit became more evident in L1-KO mice than in wild-type mice (Figure 3A) because T0901317 promoted biliary cholesterol secretion equally in both wild-type and L1-KO mice (Table 2), and yet this bile-originated cholesterol cannot be reabsorbed in L1-KO mice, unlike in wild-type mice. It is possible that this deficit in cholesterol balance specifically counterbalances LXR activation of lipogenic genes, and as a result, triglyceride is reduced in the liver of T0901317-treated L1-KO mice.

One could argue that T0901317 absorption might have been impaired in L1-KO mice so that it failed to raise plasma HDL-C and hepatic triglyceride in these animals. We do not favor this argument because LXR activation by T0901317: (1) promoted biliary cholesterol secretion equally in mice of both genotypes (Table 2); (2) increased the intestinal expression of its target ABCA1x8.8-fold in L1-KO mice versus 3.9-fold in wild-type mice (Figure 2C); and (3) caused a greater fecal loss of cholesterol in L1-KO mice compared with vehicle-treated L1-KO mice (Figure 3A), which could only be explained by T0901317 promoting excretion of endogenous cholesterol rather than inhibiting absorption of exogenous cholesterol because the same amount of cholesterol was fed to animals and the intestinal cholesterol absorption was disrupted in L1-KO mice. To promote excretion of endogenous cholesterol, T0901317 has to be absorbed into the body. Additionally, we measured by high-performance liquid chromatography (HPLC) the relative level of T0901317 that was accumulated in the pooled samples of plasmas used for lipid analyses. Both wild-type and L1-KO mice accumulated the same level of T0901317 in the plasma (data not shown). When T0901317 was administered by intraperitoneal injection, less response in terms of plasma lipoprotein cholesterol increase was also seen in L1-KO versus wild-type mice (supplemental Figure 1). It is unknown why stool output was reduced in T0901317-treated mice and in L1-KO versus wild-type mice (Figure 3C). It appears that stool output is negatively correlated with the amount of free cholesterol in the gut lumen. L1-KO mice do not absorb cholesterol, resulting in increased luminal cholesterol and reduced stool output. T0901317-treated L1-KO mice have even more cholesterol in the lumen as a result of increased biliary cholesterol secretion, thus further reducing stool output. It is tempting to hypothesize that a reduction in stool output may protect against excessive fecal loss of cholesterol, an essential structural component of mammalian cells.

The fact that the majority of cholesterol was converted to coprostanol in T0901317-treated L1-KO mice (Figure 3B) may be a result of substrate-driven activation of cholesterol-reducing bacteria in the gut lumen. It has been reported that the growth of a cholesterol-reducing bacterium requires the presence of cholesterol in the medium. The conversion of cholesterol to coprostanol may protect the intestine against cytotoxicity of free cholesterol that overaccumulates in the gut lumen of T0901317-treated L1-KO mice.

Collectively, this study unraveled a predominant role of intestinal cholesterol absorption in the biogenesis of HDL-C induced by LXR activation. As proposed by Kruit et al, in addition to liver, intestine may serve as another target for pharmacological manipulation of plasma HDL-C. Because inhibition of cholesterol absorption by ezetimibe, a potent cholesterol absorption inhibitor that targets NPC1L1, lowers plasma LDL cholesterol, targeting intestinal cholesterol absorption may produce a dual effect on lipoprotein cholesterol metabolism: (1) its inhibition lowers plasma LDL cholesterol, the offending agent for coronary heart disease, and (2) its presence is essential for some compounds to raise plasma HDL-C, the defending agent against coronary heart disease.

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Disclosures
None.

References


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Niemann-Pick C1-like 1 is required for LXR agonist to raise plasma HDL cholesterol in mice

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Online Supplemental Methods

Animals and Diets
L1-KO mice of C57BL/6 genetic background were generated as described previously.¹ Wild-type C57BL/6N mice were purchased from Harlan. All mice were housed in a specific pathogen-free animal facility in plastic cages under a temperature-controlled room (22 °C) with a daylight cycle from 6AM to 6PM. The mice were fed ad libitum a cereal-based rodent chow diet unless stated otherwise and had free access to water.

Analytical Procedures
The plasma was analyzed for total and free cholesterol, phospholipids and triglyceride by using the Cholesterol/HP (Roche), Free cholesterol C (Wako), Phospholipids (Wako), and Triglycerides/GB (Roche) enzymatic assay kits, respectively. Plasma lipoprotein distribution was determined by fast phase liquid chromatography (FPLC).²

For analysis of hepatic lipid content, the lipids were extracted from ~100 mg of liver tissues and quantified enzymatically as described previously.³
Bile samples were collected by the common bile duct cannulation as we have described previously. The only modification was that we ligated the cystic bile duct and removed the gallbladder in this study. Lipids were extracted as described from 10-15 µl of bile sample containing 10.3 µg of 5α-cholestane as an internal standard. Biliary cholesterol concentrations were determined by the gas chromatography. The concentration of phospholipids and bile acids were determined enzymatically.

**Fecal neutral sterol excretion**

After being gavaged with T0901317 for 4 days, each mouse was individually housed in a cage with a wire floor. The feces were collected for 3 days during which T0901317 was continued. The fecal samples were then processed for the measurement of fecal neutral sterol excretion as described previously.

**RNA Isolation and Quantitative Real-time PCR (qPCR)**

Total RNA was extracted from ~100 mg of tissues by using RNA Stat-60 kit (Tel-Test Inc., Friendswood, TX). The qPCR was performed as described previously. The sequences of all the primers used for qPCR have been published previously as supplementary materials.

**Preparation of Tissue Membrane Proteins and Immunoblot Analysis**

Tissue membrane proteins were prepared exactly as described previously. The protein concentrations of tissue membranes were determined by using bicinchoninic acid (BCA) Kit (Pierce). An equal amount of membrane protein from each sample was pooled in each group. The pooled membrane proteins (50 µg) were fractionated on 8% polyacrylamide gel in the
presence of sodium dodecyl sulfate. For quantification of plasma apoE levels, plasma was diluted with saline (1:200 dilutions). 14 µl of diluted plasma samples were fractioned on 12% polyacrylamide gel in the presence of sodium dodecyl sulfate. Proteins were then transferred to Hybond-C Extra nitrocellulose filter for immunoblotting as described previously. Antibodies to apoE, ABCG5, ABCA1, scavenger receptor class B type I (SR-BI), low density lipoprotein receptor (LDLR) and receptor associated protein (RAP) have been described previously.

References


5. Temel RE, Lee RG, Kelley KL, Davis MA, Shah R, Sawyer JK, Wilson MD, Rudel LL. Intestinal cholesterol absorption is substantially reduced in mice deficient in both ATP-


Figure legends

Supplemental figure 1: L1-KO mice are less responsive to T0901317: Two-month-old C57BL/6 male WT and L1-KO mice on regular chow diet were administered by IP with 25 mg/kg BW of T0901317 for 7 days. Plasma samples were collected after a 4 h fast and an equal amount of plasma from each mouse in each group (n = 3) was pooled. The same amount of pooled plasma was subjected to FPLC for monitoring lipoprotein cholesterol distribution. A greater increase in plasma lipoprotein cholesterol was seen in WT versus L1-KO mice.
Fig. S1

- WT-T
- L1-KO-T

Cholesterol/Response (mv)

Retention Time (Min)

- VLDL
- LDL
- apoE HDL
- HDL