Liver-Specific Phospholipid Transfer Protein Deficiency Reduces High-Density Lipoprotein and Non–High-Density Lipoprotein Production in Mice

Amirfarbd Yazdanyar, Wei Quan, Weijun Jin, Xian-Cheng Jiang

Objective—The liver is one of the critical organs for lipoprotein metabolism and a major source for phospholipid transfer protein (PLTP) expression. The effect of liver-specific PLTP deficiency on plasma lipoprotein production and metabolism in mice was investigated.

Approach and Results—We created a liver-specific PLTP-deficient mouse model. We measured plasma high-density lipoprotein (HDL) and apolipoprotein B (apoB)-containing lipoprotein (or non-HDL) levels and their production rates. We found that hepatic ablation of PLTP leads to a significant decrease in plasma PLTP activity, HDL lipids, non-HDL lipids, apoAI, and apoB levels. In addition, nuclear magnetic resonance examination of lipoproteins showed that the deficiency decreases HDL and apoB-containing lipoprotein particle numbers, as well as very low-density lipoprotein particle size, which was confirmed by electron microscopy. Moreover, HDL particles from the deficient mice are lipid-poor ones. To unravel the mechanism, we evaluated the apoB and triglyceride production rates. We found that hepatic PLTP deficiency significantly decreases apoB and triglyceride secretion rates. To investigate the role of liver PLTP on HDL production, we set up primary hepatocyte culture studies and found that the PLTP-deficient hepatocytes produce less nascent HDL. Furthermore, we found that exogenous PLTP promotes nascent HDL production through an ATP-binding cassette A1-mediated pathway.

Conclusions—Liver-specific PLTP deficiency significantly reduces plasma HDL and apoB-containing lipoprotein levels. Reduction of production rates of both particles is one of the mechanisms. (Arterioscler Thromb Vasc Biol. 2013;33:2058-2064.)

Key Words: atherosclerosis ■ high-density lipoproteins ■ liver ■ phospholipid transfer protein ■ very low-density lipoproteins

Phospholipid transfer protein (PLTP) belongs to a family of lipid transfer/lipoprotein-binding proteins, including cholesteryl ester transfer protein, lipopolysaccharide-binding protein, and bactericidal/permeability increasing protein. It is a monomeric protein of 81 kDa. Besides transferring phosphatidylcholine, PLTP also efficiently transfers sphingomyelin, cholesterol, diacylglycerol, and apoAI and apoB levels. In addition, nuclear magnetic resonance examination of lipoproteins showed that the deficiency decreases HDL and apoB-containing lipoprotein particle numbers, as well as very low-density lipoprotein particle size, which was confirmed by electron microscopy. Moreover, HDL particles from the deficient mice are lipid-poor ones. To unravel the mechanism, we evaluated the apoB and triglyceride production rates. We found that hepatic PLTP deficiency significantly decreases apoB and triglyceride secretion rates. To investigate the role of liver PLTP on HDL production, we set up primary hepatocyte culture studies and found that the PLTP-deficient hepatocytes produce less nascent HDL. Furthermore, we found that exogenous PLTP promotes nascent HDL production through an ATP-binding cassette A1-mediated pathway.

Conclusions—Liver-specific PLTP deficiency significantly reduces plasma HDL and apoB-containing lipoprotein levels. Reduction of production rates of both particles is one of the mechanisms. (Arterioscler Thromb Vasc Biol. 2013;33:2058-2064.)

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Received on: April 1, 2013; final version accepted on: June 24, 2013.
The online-only Data Supplement is available with this article at http://atvb.ahajournals.org/lookup/suppl/doi:10.1161/ATVBHA.113.301628/-/DC1.
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Arterioscler Thromb Vasc Biol is available at http://atvb.ahajournals.org
DOI: 10.1161/ATVBHA.113.301628
lipoprotein (VLDL) overproduction.\textsuperscript{17} Associations of plasma PLTP activity with elevated apoB levels have been found in humans as well.\textsuperscript{18} Dr Largrost’s group\textsuperscript{19} found that human PLTP transgenic rabbits showed a significant increase of BLp cholesterol in the circulation. Nevertheless, the surprising finding that PLTP affects BLp secretion from the liver has remained unexplained. We believe that PLTP activity is involved in promoting BLp lipidation because PLTP activity and triglyceride enrichment are 2 factors for PLTP-mediated HDL enlargement,\textsuperscript{10,11} a process similar to the second step of BLp lipidation.\textsuperscript{20} To address the impact of liver-expressed PLTP on lipoprotein metabolism, we created a mouse model that expresses PLTP acutely and specifically in the liver on a PLTP-null background. We found that liver PLTP expression dramatically increases plasma non-HDL cholesterol, non-HDL phospholipid, and triglyceride levels, with a slight increase on plasma HDL lipids, compared with the controls.\textsuperscript{21}

To further investigate the impact of liver PLTP on nascent HDL and BLp production, we prepared a liver-specific PLTP gene KO mouse model. We found that liver-specific PLTP deficiency significantly reduces both HDL and non-HDL levels. We also explored the possible mechanisms.

Materials and Methods

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

Results

Liver PLTP Deficiency Decreases Plasma PLTP Activity

We prepared homozygous PLTP-Flox mice with a C57BL/6 genetic background. However, the Flox mice have no PLTP activity in the circulation because of Neo cassette insertion in intron 3.\textsuperscript{21} Because the Neo cassette is double-flanked by both LoxP and flipase recognition target sequences (Figure 1A), we crossed our PLTP-Flox mice with flipase-transgenic mice. Flipase recognizes the flipase recognition target sequences.\textsuperscript{22} Through this method, we prepared the PLTP-Flox/flipase transgenic (ΔNeo) mice. We found that the ΔNeo mice have normal plasma PLTP activity (Figure 1B), as well as plasma cholesterol and phospholipid levels (Figure 1A and IB in the online-only Data Supplement).

Next, we took the advantage of the Cre/LoxP system to delete PLTP exons 2 and 3, which are flanked with LoxP sequences. We injected adenovirus-associated virus (AAV)-Cre and AAV-LacZ (controls) into the ΔNeo mice. Because Cre expression was driven by the liver-specific thyroxine binding globulin promoter,\textsuperscript{23} the PLTP deletion occurred specifically in the liver (Figure 1C). Moreover, AAV-Cre-mediated hepatic PLTP ablation resulted in \textasciitilde25% reduction of plasma PLTP activity in comparison with the AAV-LacZ controls (Figure 1D), indicating that liver-expressed PLTP makes a significant contribution to the PLTP activity in the blood. Furthermore, PLTP activity in AAV-Cre–injected livers is significantly lower than that of controls (Figure 1E).

PLTP Hepatic Deficiency Significantly Decreases Both Plasma HDL and Non-HDL Lipid Levels

AAV-Cre–treated PLTP-Flox female animals significantly decreased plasma levels of cholesterol (21%; \textit{P}<0.01), phospholipids (22%; \textit{P}<0.05), and triglyceride (32%; \textit{P}<0.05), as compared with AAV-LacZ control group (Table 1). We also measured lipid levels in HDL and non-HDL fractions. Our data showed that liver PLTP depletion significantly decreases not only HDL cholesterol (20%; \textit{P}<0.05) and HDL phospholipid (17%; \textit{P}<0.05) but also non-HDL cholesterol (29%; \textit{P}<0.01) and phospholipid (35%; \textit{P}<0.01) levels (Table 1).

Plasma lipid distributions were also examined by fast protein liquid chromatography using pooled plasma. We observed that plasma cholesterol levels are decreased in HDL and non-HDL fractions from the female-deficient mice, as compared with controls (Figure 2A). This is also true for total phospholipid distribution (Figure 2B) and that of triglyceride (Figure 2C). The same phenomena were also observed in AAV-Cre–treated ΔNeo male mice, as compared with male controls (Figure II A–IC in the online-only Data Supplement).

Next, we assessed plasma apolipoprotein levels by reducing SDS-PAGE and found that the PLTP-deficient mice have a marked decrease of total apoB (60%; \textit{P}<0.01; Figure 2D) and apoAI (35%; \textit{P}<0.01; Figure 2E), as compared with the control group. This suggests that PLTP deficiency in the liver has impact on both apoAI-containing lipoprotein (HDL) and BLp (non-HDL) levels.

To investigate the mechanisms responsible for the reduced triglyceride and apoB levels in liver-specific PLTP-deficient mouse plasma, we examined the VLDL production rates in vivo. Both AAV-Cre and AAV-LacZ mice were simultaneously injected with \textsuperscript{35}S-methionine to label apoB, \textsuperscript{14}C-oleic acid to label triglyceride, and poloxamer 407 to block the clearance of VLDL from the circulation. We collected plasma 120 minutes after injection and isolated plasma VLDL by ultracentrifugation (density, 1.006 g/mL). We found that both \textsuperscript{35}S-apoB and \textsuperscript{14}C-triglyceride levels were significantly decreased in the VLDL from the deficient mice, as compared with that of the controls (Figure 2F and 2G). This suggests that liver PLTP deficiency prevents VLDL secretion.

To further investigate the effect of hepatic PLTP on lipoprotein metabolism, we used nuclear magnetic resonance to measure total particle number, size, and lipid composition of VLDL, LDL, and HDL. We found that the lack of PLTP in the liver decreases the size and particle number of VLDL particles (Table 2). In addition, these particles have less triglyceride in comparison with controls (Table 2). In LDL and HDL, the reduction in particle numbers was also observed; however, we did not detect any noticeable size differences between the deficient animals and controls (Table 2). Moreover, HDL from the deficient animals has less cholesterol compared with controls (Table 2). To confirm the HDL results, we isolated HDL from the deficient and control mouse plasma by ultracentrifugation. We measured cholesterol and phospholipid levels in the HDLs and found that, indeed, the HDL particles from the deficient mice is lipid-poor ones (Table 1).

We next sought to examine lipoprotein particles under electron microscopy. As shown in Figure 3, the deficient mice have smaller VLDL particles. After counting 100 particles, the average sizes for VLDL are 52±10 and 37±5 nm (controls versus KO; \textit{P}<0.05). However, for LDL and HDL particles, there is no significant size difference between the 2 groups.
These results confirmed what we have observed in nuclear magnetic resonance analysis (Figure 3). These results confirmed what we have observed in nuclear magnetic resonance analysis (Table 2).

**PLTP Promotes Nascent HDL Production**

To further investigate the mechanisms behind the reduction of apoAI and HDL lipids in plasma, we examined apoAI production. Both AAV-Cre and AAV-LacZ mice were simultaneously injected with [35S]-methionine to label proteins. We collected plasma and isolated HDL by ultracentrifugation (density, 1.21 g/mL). We then separated [35S]-apoAI on SDS-PAGE and found that there is no significant change in [35S]-apoAI levels between the 2 groups (Figure IIIA and IIIB in the online-only Data Supplement). We also examined ABCA1 and SR-BI levels in liver homogenates and found no significant change either (Figure IIIC–IIIE in the online-only Data Supplement).

We next sought to measure nascent HDL production directly. Primary hepatocytes from PLTP KO and control mice were isolated and labeled with 3H-cholesterol. The cells were then incubated with 50 µg/mL human apoAI. The medium was collected after 5-hour incubation. Non-HDL and HDL were separated by ultracentrifugation. The radioactivity in HDL fraction was determined by liquid scintillation counting. We found that PLTP deficiency significantly reduces hepatocyte nascent HDL production (Figure 4A).

We further isolated primary hepatocytes from PLTP KO mice and labeled them with 3H-cholesterol. The cells were then incubated with 50 µg/mL human apoAI together with active recombinant PLTP (rPLTP; 1 µg/mL) or heat-inactivated rPLTP. The medium was collected, and non-HDL and HDL were separated. The radioactivity in HDL fraction was determined by liquid scintillation counting. We found that exogenous active rPLTP significantly promoted hepatocyte nascent HDL production (Figure 4B).

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It has been reported that exogenous PLTP can promote removing cholesterol and phospholipids from macrophages through the ABCA1 pathway. Although PLTP has no effect on lipid efflux from fibroblasts isolated from ABCA1-deficient patients, hepatocytes may react differently. To evaluate whether PLTP-mediated nascent HDL production is ABCA1 dependent or not, we next isolated primary hepatocytes from liver-specific ABCA1 KO mice (Figure IV A in the online-only Data Supplement) and labeled them with 3H-cholesterol. We then incubated them with apoAI together with active rPLTP or inactive rPLTP. We did not find significant difference in nascent HDL production (Figure IVB in the online-only Data Supplement).

### Table 1. Plasma Lipid Measurement

<table>
<thead>
<tr>
<th></th>
<th>BLp-C</th>
<th>BLp-PL</th>
<th>HDL-C</th>
<th>HDL-PL</th>
<th>TG</th>
<th>HDL-C</th>
<th>HDL-PL</th>
</tr>
</thead>
<tbody>
<tr>
<td>AAV-LacZ</td>
<td>28±3</td>
<td>55±6</td>
<td>80±4</td>
<td>167±8</td>
<td>75±5</td>
<td>0.23±0.02</td>
<td>0.42±0.01</td>
</tr>
<tr>
<td>AAV-Cre</td>
<td>21±3</td>
<td>36±9*</td>
<td>64±2*</td>
<td>138±11*</td>
<td>51±4*</td>
<td>0.16±0.01*</td>
<td>0.31±0.03*</td>
</tr>
</tbody>
</table>

AAV indicates adenovirus-associated virus; BLp, apolipoprotein B–containing lipoprotein; HDL-C, high-density lipoprotein cholesterol; PL, phospholipid; and TG, triglyceride.

All mice are female. Plasma samples were collected after fasting. Values represent mean±SD (n=6; *P<0.05).

HDL particles were separated by ultracentrifugation (density, <1.21 g/mL). All values are normalized on the basis of protein levels of HDL fractions and presented as mean±SD (n=3; *P<0.05).
Finally, we performed cross-linking experiment as described in Materials and Methods in the online-only Data Supplement to prove that there is an interaction between PLTP and ABCA1. After protein blotting, we found that there are 2 detectable ABCA1 and PLTP bands, 1 with 260 kDa (complex 1) and 1 with >500 kDa (complex 2; Figure 4C), indicating that rPLTP and ABCA1 are close enough to be cross-linked and suggesting that PLTP can interact with ABCA1, thus promoting nascent HDL formation.

### Discussion

AAV-Cre/LoxP, adenovirus-Cre/LoxP, Mx1-Cre/LoxP (Cre transgene is controlled by the interferon-inducible Mx1 promoter), and albumin-Cre/LoxP (Cre transgene expression is controlled by liver-specific albumin promoter) are 4 approaches for liver-specific gene KO mouse preparation. The former 3 approaches eliminate the gene in the adult mice, whereas the latter eliminates the gene in the early stage of the life. As a pilot study of a drug intervention, AAV-Cre, adenovirus-Cre, and Mx1-Cre approaches are better than albumin-Cre approach because they mimic drug intervention. PLTP is a potential drug target and we chose to block its activity in the adult mice.

There are also some important differences among AAV-Cre, adenovirus-Cre, and Mx1-Cre approaches. The concern

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**Table 2. Nuclear Magnetic Resonance Analysis of Lipoprotein Particles**

<table>
<thead>
<tr>
<th></th>
<th>Mean Particle Size, nm</th>
<th>Particle Concentration, mmol/L</th>
<th>Calculated Lipid, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>VLDL</td>
<td>LDL</td>
<td>HDL</td>
</tr>
<tr>
<td>AAV-LacZ</td>
<td>52.8</td>
<td>22.8</td>
<td>9.4</td>
</tr>
<tr>
<td>AAV-Cre</td>
<td>37.1</td>
<td>21.1</td>
<td>9.8</td>
</tr>
</tbody>
</table>

All mice are female. Values represent mean of pooled (n=6) samples. AAV indicates adenovirus-associated virus; HDL-C, high-density lipoprotein cholesterol; LDL, low-density lipoprotein; TG, triglyceride; and VLDL, very low-density lipoprotein.

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**Figure 2.** Plasma lipid distribution, apolipoprotein, and non–high-density lipoprotein (HDL) production measurements. Plasma lipid distributions were analyzed by fast protein liquid chromatography. A 250-μL aliquot of pooled plasma (from 5 male animals) was loaded onto the columns and eluted with Tris buffer (50 mmol/L, pH 7.4) at a constant flow rate of 0.35 mL/min. A, Cholesterol distribution; B, phospholipid distribution; C, triglyceride distribution; D and E Plasma (0.2 μL) was separated by 4% to 15% SDS-PAGE and immunoblotted with polyclonal antibodies against apolipoprotein B (apoB) and apoA1. The results were quantified with ImageJ software. D, apoB Western blot and total apoB quantification; E, apoA1 Western blot and quantification. ApoB-containing triglyceride-rich lipoprotein production in vivo was measured as described in Materials and Methods in the online-only Data Supplement. F, Total [35S]-apoB in very low-density lipoprotein (VLDL) and quantification. Total [14C]-triglyceride in VLDL and quantification. Values are mean±SD (n=5; *P<0.01). AAV indicates adenovirus-associated virus.
Liver PLTP deficiency prevents BLp production. Liver is one of the major tissues for lipoprotein production and PLTP expression. Our previous work showed that liver PLTP expression with a PLTP-null background dramatically promotes BLp production, through more lipidation and secretion. Our finding together with previous findings provides the concept that PLTP-mediated VLDL production per se is one of the driving forces for plasma lipoprotein metabolism. In this context, our liver-specific PLTP-deficient mouse is another useful tool to further elucidate the effect of PLTP on VLDL production in the liver. We found that hepatic PLTP deficiency significantly decreases plasma non-HDL lipid levels (Table 1) and BLp production rates (Figure 2F and 2G). This may be related to (1) less apoB particle lipidation and secretion and (2) intracellular reactive oxygen species–triggered apoB post-endoplasmic reticulum presecretory proteolysis.

Our previous study also showed that liver PLTP expression with a PLTP-null background has marginal effect on HDL lipid and apoAI levels. However, in the current study, we found that liver PLTP deficiency (or extrahepatic PLTP expression) decreases HDL lipid and apoAI levels (Table 1; Figure 2E). This cannot be simply explained by the reduction of plasma PLTP activity (≈25%; Figure 1D) because heterozygous systemic PLTP deficiency (45% reduction of plasma PLTP activity) has no observable HDL lipid phenotypes. It is possible that there are multiple PLTP pools which have different types of impact on HDL metabolism.

Liver PLTP deficiency–mediated reduction of HDL levels may be related to lower HDL production from the liver. Nascent HDL was found in mesenteric lymph and hepatic perfusates, where it may arise as a result of PLTP-mediated lipid transfer from cellular plasma membrane into the small intestine–secreted and liver-secreted apoAI. ABCA1 mediates the rate-controlling step in HDL particle formation by promoting the efflux of cholesterol and phospholipids to apoAI. Overexpression of hepatic ABCA1 raises HDL cholesterol levels, and liver-specific deletion of ABCA1 dramatically decreases plasma HDL cholesterol in mice. Exogenous PLTP can promote removing cholesterol and phospholipids from macrophages by the ABCA1 pathway. In contrast, PLTP had no effect on lipid efflux from fibroblasts isolated from a patient with Tangier disease.

The same group of researchers also indicated that an amphipathic helical region (aa144–aa163) of PLTP is critical for ABCA1-dependent cholesterol efflux, and they found that PLTP can stabilize ABCA1 on cell plasma membrane.

In line with these reports, we found that liver PLTP is a significant player in nascent HDL production through ABCA1 pathway. On the basis of our observation, ablation of PLTP in the liver has no effect on apoAI production (Figure IIIA and IIIB in the online-only Data Supplement) and ABCA1 protein levels in tissue homogenates (Figure IIC and IID in the online-only Data Supplement). However, PLTP deficiency significantly reduces nascent HDL production (Figure 4A), and exogenous active rPLTP significantly enhances hepatocyte nascent HDL production (Figure 4B). Furthermore, we found that this PLTP-mediated effect requires the presence of ABCA1 because rPLTP has no effect in HDL production in ABCA1-deficient primary hepatocytes (Figure IVA and IVB).
in the online-only Data Supplement). We believe that, as proposed in macrophages, PLTP may function to (1) stabilize liver ABCA1 and (2) shuttle lipids between cells and existing HDL particles (formed first through ABCA1 action).

Hepatic PLTP deficiency-mediated reduction of HDL levels might be related to less VLDL production. PLTP can transfer lipids from the surface of BLp into HDL. Systemic PLTP deficiency completely blocks this transfer activity. After mature BLp secreted into the blood and lipoprotein lipase–mediated triglyceride hydrolysis, the core of BLp shrinks, and the redundant surface constituents (sphingomyelin and phosphatidylcholine, as well as free cholesterol) can be the substrates of PLTP, transferring from BLp to HDL.

PLTP may have an antiatherogenic function because it promotes nascent HDL production. However, recent researches challenge the concept that rising of plasma HDL will uniformly translate into reductions in coronary heart diseases. HDL particles are heterogeneous in size and composition. Understanding the origination of HDL and characterization of HDL, all subclasses are as important as its plasma concentration in leading to atherosclerotic lesion development. In this study, we showed that PLTP activity promotes nascent HDL production (Figure 4A and 4B), through the interaction between PLTP and ABCA1 (Figure 4C). Whether this PLTP function is antiatherogenic or proatherogenic deserves further investigation. However, PLTP overexpression promotes and deficiency prevents atherosclerosis in mice.

Taking together the results from the current study and our previous one, we proposed a model for PLTP-mediated HDL and non–HDL production (Figure 4D). We believe that (1) PLTP may be involved in the second step of BLp lipidation, promoting the fusion of primordial BLp and triglyceride/sphingomyelin/phosphatidylcholine-rich lipid droplets; (2) PLTP may promote ABCA1-mediated nascent HDL production through stabilizing ABCA1 and shunting lipids between cells and existing HDL particles; and (3) PLTP may transfer BLp surface components into HDL, after lipoprotein lipase–mediated core triglyceride hydrolysis.

Acknowledgments
We thank Drs John Albers and Simona Vuletic from University of Washington, Seattle, for providing human phospholipid transfer protein antibody.

Sources of Funding
This work was supported by National Institutes of Health grants HL69817 and VA Merit 000000-01.

Disclosures
None.

References

**Significance**

Our knowledge of atherosclerosis is still limited, and more extensive investigation must provide better understanding of this disease. Exploring the effect of phospholipid transfer protein (PLTP) on lipid metabolism is such an investigation because PLTP is a risk factor for the development of the disease. Apolipoprotein B-containing lipoprotein and high-density lipoprotein production are 2 processes that are closely related with atherosclerosis. The current study is the first one to indicate that liver-specific PLTP deficiency can suppress both apolipoprotein B-apolipoprotein and high-density lipoprotein production. PLTP may accelerate apolipoprotein B lipidation in the endoplasmic reticulum lumen of hepatocytes by promoting fusion of lipid droplet and primordial apolipoprotein B particle. PLTP may increase hepatocyte nascent high-density lipoprotein production by promoting ATP-binding cassette A1-mediated lipid efflux.
Liver-Specific Phospholipid Transfer Protein Deficiency Reduces High-Density Lipoprotein and Non-High-Density Lipoprotein Production in Mice
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Arterioscler Thromb Vasc Biol. 2013;33:2058-2064; originally published online July 11, 2013; doi: 10.1161/ATVBAHA.113.301628

Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272 Greenville Avenue, Dallas, TX 75231
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Print ISSN: 1079-5642. Online ISSN: 1524-4636

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Supplement

**Supplemental Fig. I.** Plasma lipid measurements. Panel A, plasma total cholesterol levels. Panel B, plasma total phospholipids in mice. All mice are male. N=5.

**Supplemental Fig. II.** Plasma Lipid distribution. Plasma lipid distributions were analyzed by fast protein liquid chromatography (FPLC). A 250-μl aliquot of pooled plasma (from five male animals) was loaded onto the columns and eluted with Tris buffer (50 mM, pH 7.4) at a constant flow rate of 0.35 ml/min. Panel A, cholesterol distribution. Panel B: phospholipid distribution. Panel C: triglyceride distribution.

**Supplemental Fig. III.** ApoA-I production and ABCA1/SR-BI Western blot analyses. Panel A, apoA-I production measurement. Mice were injected with [35S] methionine (200μCi) to label total proteins. Plasma (150μl) was collected 120 minutes after injection, and HDL was isolated from the plasma by ultracentrifugation (1.21 g/ml). The same volume of isolated HDL (250μl) was loaded on 4-15% gradient gel, and [35S] apoA-I was separated by SDS-PAGE. Incorporation of 35S into ApoA-I was assessed with a Fuji Bio-Imaging Analyzer. Panel B, quantification results of apoA-I production. Panel C, Western blot of ABCA1 and SR-BI using liver homogenates. The loading control was GAPDH. Panel D, quantification results of ABCA1. Panel E, quantification results of SR-BI. Values are mean ± SD, n=3-4,
Supplemental Fig. IV. rPLTP has no effect on nascent HDL production in ABCA1 deficiency hepatocytes. Adenovirus (AdV)-Cre was injected (i.v.) into ABCA1-Flox mice. Liver homogenate was prepared after 5 days of infection. Panel A, ABCA1 Western blot. Panel B, nascent HDL production using rPLTP. N=3.
Supplemental Fig. I. Yazdanyar et al.

![Bar graph showing cholesterol levels](image)

*Significant difference

![Bar graph showing phospholipid levels](image)

*Significant difference
Supplemental Fig. III. Yazdanyar et al.

A

$^{35}$S-ApoA-I → AAV-LacZ → AAV-Cre

B

Apo-AI levels (% of AAV-LacZ)

0 50 100 150

AAV-LacZ AAV-Cre

C

AAV-LacZ → AAV-Cre

ABCA1

SR-BI

GAPDH

D

ABCA1 levels (% of AAV-LacZ)

0 50 100 150

AAV-LacZ AAV-Cre

E

SR-BI levels (% of AAV-LacZ)

0 50 100 150

AAV-LacZ AAV-Cre
Supplemental Fig. IV. Yazdanyar et al.

A

[Image of Western blot results showing ABCA1 and β-Actin expression with Ad-Cre and Control groups.]

B

[Bar graph showing [3H] DPM of HDL fraction with NS indicated between HI rPLTP and rPLTP groups.]
**Materials and Methods**

**PLTP-Flox mouse preparation.** To prepare PLTP-Flox mice, a 9.16kb region used to construct the targeting vector was first subcloned from a positively identified C57BL/6 (RPCI23) BAC clone. The region was designed so that the short homology arm extended about 1.68kb 3’ to exon 3, and the long homology arm extended about 6.85kb long 5’ to exon 2. The loxP and FRT double-flanked Neo cassette was inserted on the 3’ side of exon 3, and the single loxP site was inserted into the 5’ side of exon 2 (Fig. 1A). All mice used in this study were aged 12-16 weeks, with a C57BL/6J genetic background. They were fed a rodent chow diet (Purina Laboratory Rodent Chow 5001). All animal procedures were approved by the SUNY Downstate Medical Center Animal Care and Use Committee.

**Liver-specific PLTP deficient mouse preparation.** We first deleted the Neo cassette from the PLTP Flox gene construct using Flp transgenic mice from Jackson Laboratory, thus created ΔNeo mice (Fig. 1A). Since exon 2, which contains starting codon, and exon 3 are flanked with LoxP sequences (Fig. 1A), both exons were eliminated specifically in the liver, by using adenovirus associated virus (AAV)-mediated expression of Cre recombinase (AAV-Cre), which recognizes the LoxP sequences. We injected (i.p.) AAV-Cre (1 X 10^12) into the ΔNeo mice. Since the Cre recombinase expression was driven by the liver-specific thyroxine binding globulin (TBG) promoter, the AAV approach resulted in sustained Cre expression in the liver for at least 20 weeks.
**Lipid and lipoprotein assays.** The total cholesterol, total phospholipids, and triglyceride in plasma were assayed by enzymatic methods. Lipoprotein profiles were obtained by fast protein liquid chromatography (FPLC), using a Sepharose 6B column.

**Apolipoprotein measurement.** Plasma apoB and apoA-I levels were determined, as previously described. Briefly, 0.2 µl of plasma were separated by 4-15% SDS gel electrophoresis, and immunoblotted with polyclonal antibodies against apoB (Abcam), and apoA-I (Abcam).

**VLDL production measurement (in vivo).** Mice were injected with [35S] methionine (200µCi) to label apoB, [14C]-oleic acid (100 µCi) to label triglyceride, and with Poloxamer 407 to block the clearance of VLDL from circulation. Plasma (150µl) was collected 120 minutes after injection and VLDL was isolated from the plasma by ultracentrifugation. The same volume of isolated VLDL (250µl) was loaded on 4-15% gradient gel, and apoB was separated by SDS-PAGE. Incorporation of 35S into ApoB48 and ApoB100 was assessed with a Fuji Bio-Imaging Analyzer. Lipids in isolated VLDL were extracted by the Folch method, and separated by thin-layer chromatograph (TLC). The amount of radioactivity in the triglyceride fraction was measured by a liquid scintillation counter.

**Nuclear magnetic resonance (NMR) analysis of lipoprotein size.** We measured the lipoprotein subclass profiles on aliquots (500 µl) of EDTA plasma by proton NMR
spectroscopy at LipoScience, Inc. (Raleigh, NC), on a fee-for-service basis, as we did in our previously study.

**Electron microscopy.** Negative-stain electron microscopy was conducted as described before.

**Liver perfusion and primary hepatocyte culture.** Primary hepatocytes were isolated as previously described. Briefly, liver cells were isolated from 10 week-old male PLTP KO mice by collagenase perfusion. The isolated hepatocytes were dispensed on a 6-well plate coated with collagen I (Biocoat, Becton Dickinson, Bedford, MA). After 60 min incubation at 37 °C under 5% CO2, the unattached cells were removed and the cells were washed with PBS. Then, the cells received fresh complete medium and incubated for 2h before any treatment.

**Nascent HDL production.** The cultured primary hepatocytes were loaded with LDL (50 mg protein/ml) that had been pre-incubated with 5 mCi of $[^{3}H]$-cholesterol in DMEM with 1% FBS along with ACAT inhibitor (10 nM oleic acid-2,6-diisopropylanilide, Cayman Chemical Co., Ann Arbor, MI) for 24 h. The next day, the cells were washed twice with pre-warmed PBS and incubated in fresh DMEM for 30 min. Then, the cells were incubated with human apoA-I (A0722, Sigma-Aldrich, St. Louis, MO) with either recombinant PLTP (rPLTP) or heat inactivated rPLTP. After 5h incubation, media were collected. The density was adjusted to 1.063 g/ml, then spun (Optima TLX Ultracentrifuge) at 980,000 rpm for 4 hours to remove apoB-containing particles. Then
the medium was adjusted to 1.21 g/ml and spun at 980,000 rpm for 4 hours. The lipid radioactivity correspondent to HDL fraction was counted and adjusted based on cellular protein concentration.

**PLTP-ABCA1 crosslinking.** Confluent primary hepatocytes were cultured in 6-well collagen coated plates. After 24 hr, the cells were washed three times with ice-cold PBS (PH 8.0) to remove amine-containing culture media and proteins from the cells. The cells were then treated with rPLTP and incubated for 1 hr at 4°C. The surface protein complexes were cross-linked with bis (sulfo succinimidyl) suberate (BS3) (Pierce, Rockford, Ill.) (the final concentration of 2.5 mM) for 30 min at room temperature. The cross-linking reaction was stopped with the addition of 50 μl of 1 M Tris-HCl as quenching solution for 15 minutes at room temperature. Cells were washed twice with ice-cold PBS and then were solubilized with 125 μl of 20 mM Tris buffer, pH 7.4, containing 1% Triton X-100, 10% glycerol, 1 mM EDTA, and protease inhibitors. Solubilized material was recovered from each well and centrifuged for 10 min at 4°C to pellet cell debris, and the supernatants were transferred to 1/5 volume of fivefold-concentrated electrophoresis sample buffer. The protein complexes were analyzed using 5% sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and were visualized by blotting against ABCA1.

**Statistical Analysis.** Each experiment was conducted at least three times. Data are typically expressed as mean ± SD. Data between two groups were analyzed by the unpaired, two-tailed Student's *t*-test, and among multiple groups by ANOVA followed by
the Student-Newman-Keuls (SNK) test. A $P$ value of less than 0.05 was considered significant.