Impact of Phospholipid Transfer Protein on Nascent High-Density Lipoprotein Formation and Remodeling

Ailing Ji, Joanne M. Wroblewski, Nancy R. Webb, Denys R. van der Westhuyzen

Objective—Phospholipid transfer protein (PLTP), which binds phospholipids and facilitates their transfer between lipoproteins in plasma, plays a key role in lipoprotein remodeling, but its influence on nascent high-density lipoprotein (HDL) formation is not known. The effect of PLTP overexpression on apolipoprotein A-I (apoA-I) lipidation by primary mouse hepatocytes was investigated.

Approach and Results—Overexpression of PLTP through an adenoviral vector markedly affected the amount and size of lipidated apoA-I species that were produced in hepatocytes in a dose-dependent manner, ultimately generating particles that were <7.1 nm but larger than lipid-free apoA-I. These <7.1-nm small particles generated in the presence of overexpressed PLTP were incorporated into mature HDL particles more rapidly than apoA-I both in vivo and in vitro and were less rapidly cleared from mouse plasma than lipid-free apoA-I. The <7.1-nm particles promoted both cellular cholesterol and phospholipid efflux in an ATP-binding cassette transporter A1–dependent manner, similar to apoA-I in the presence of PLTP. Lipid-free apoA-I had a greater efflux capacity in the presence of PLTP than in the absence of PLTP, suggesting that PLTP may promote ATP-binding cassette transporter A1–mediated cholesterol and phospholipid efflux. These results indicate that PLTP alters nascent HDL formation by modulating the lipidated species and by promoting the initial process of apoA-I lipidation.

Conclusions—Our findings suggest that PLTP exerts significant effects on apoA-I lipidation and nascent HDL biogenesis in hepatocytes by promoting ATP-binding cassette transporter A1–mediated lipid efflux and the remodeling of nascent HDL particles. (Arterioscler Thromb Vasc Biol. 2014;34:1910-1916.)

Key Words: cholesterol ■ hepatocytes ■ nascent HDL ■ phospholipid transfer protein
particles, lipid-poor apolipoprotein A-I (apoA-I) is released. The mechanism(s) involved in these processes and their exact physiological relevance remain poorly understood.

A key role for PLTP in vivo is evident in PLTP-deficient mice. These mice lack plasma transfer activities for phosphatidylcholine, phosphatidylethanolamine, phosphatidylinositol, sphingomyelin and have reduced cholesterol transfer activity. The reduced plasma PLTP activity results in markedly decreased plasma HDL lipid and apolipoproteins, indicating the importance of the transfer of triglyceride-rich lipoproteins surface components in maintaining HDL levels. Liver-specific PLTP deficiency also significantly reduces plasma HDL and apoB-containing lipoprotein levels in mice, demonstrating the importance of hepatic PLTP in maintaining the lipoprotein levels. PLTP transgenic mice that overexpress human PLTP were shown, somewhat unexpectedly, to exhibit a modest but significant decrease (30–40%) in plasma HDL cholesterol. However, despite a lower HDL concentration, plasma from transgenic animals is much more efficient in preventing the accumulation of intracellular cholesterol in macrophages than plasma from wild-type (WT) mice, suggesting that PLTP contributes to HDL formation.

The influence of PLTP on nascent HDL formation may be one of the mechanisms to explain the conflicting results in terms of the effect of PLTP on HDL levels. A role for PLTP in HDL production is suggested by the observation that primary PLTP-deficient hepatocytes produce less nascent HDL. The lipidated apoA-I secreted by PLTP-deficient hepatocytes contains less phosphatidylcholine and sphingomyelin and differs also in its phosphatidylcholine/sphingomyelin ratio and fatty acyl species composition compared with lipided apoA-I from WT hepatocytes. Several studies have demonstrated that PLTP enhances cellular cholesterol and phospholipid efflux. Treatment of cholesterol-loaded human skin fibroblasts with PLTP increases HDL binding to cells and enhances cholesterol and phospholipid efflux. PLTP stabilizes ATP-binding cassette transporter A1 (ABCA1), and the absence of endogenous PLTP impairs ABCA1-dependent efflux of cholesterol from lipid-loaded macrophages. An amphipathic helical region of the N-terminal barrel of PLTP seems critical for ABCA1-dependent cholesterol efflux.

In this study, we investigated the impact of PLTP overexpression on nascent HDL formation and remodeling by primary mouse hepatocytes. Our findings indicate that PLTP exerts significant effects on apoA-I lipidation and nascent HDL biogenesis in hepatocytes by promoting both ABCA1-mediated cholesterol efflux and nascent HDL particle remodeling.

### Materials and Methods

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

### Results

**PLTP Alters Nascent HDL Formation in Hepatocytes**

To investigate the effects of PLTP on nascent HDL formation in hepatocytes, primary hepatocytes were harvested 24 hours after administration of adenovirus encoding mouse phospholipid transfer protein (AdPLTP) to mice. After overnight culture, cells were treated with the liver X receptor agonist T0901317 (5 μmol/L) for 8 hours and then incubated for 16 hours with lipid-free human apoA-I (10 μmol/mL). Culture medium was then characterized by nondenaturing gradient gel electrophoresis (GGE) and subsequent immunoblotting for human apoA-I (Figure 1A). A marked overexpression of PLTP in hepatocytes was shown by real-time polymerase chain reaction (Figure 1B) and Western blot analysis (Figure 1C). As expected, ABCA1 was expressed in hepatocytes and the liver X receptor agonist increased ABCA1 expression. The expression of PLTP in hepatocytes did not significantly alter ABCA1 expression (Figure 1 in the online-only Data Supplement).

Compared with the level of apoA-I lipidation with hepatocytes from AdNull control mice, the spectrum of nascent HDL particles produced by hepatocytes with PLTP overexpression...
was markedly changed. Overexpression of PLTP in hepatocytes affected the levels and the species of nascent HDL particles produced by hepatocytes. Although the formation of smaller nascent HDLs was reduced by PLTP, the formation of the larger HDLs was increased. These data demonstrate for the first time that PLTP affects nascent HDL biogenesis in addition to its role in mature HDL remodeling.

**PLTP Remodels Nascent HDL Particles In Vitro**

PLTP has been shown to remodel HDL by facilitating transfer of phospholipids between lipoprotein particles. PLTP can also serve as a putative fusion factor to enlarge HDL particles. However, the role of PLTP on nascent HDL remodeling is not clear. To study the effect of PLTP on preexisting nascent HDL, nascent HDL particles were generated by incubating primary hepatocytes with 5 μg/mL human 125I-apoA-I for 24 hours. Media containing these nascent HDL particles were then incubated in vitro with PLTP containing conditioned medium produced by African green monkey kidney fibroblast-like cells (COS-7) transfected with AdPLTP. The sample was subjected to the nondenaturing GGE and visualized by autoradiography. As shown in Figure 2A, PLTP efficiently remodeled nascent HDLs in vitro in a dose-dependent manner, ultimately generating small nascent HDL (sn-HDL) particles, which were <7.1 nm but slightly larger than lipid-poor apoA-I. Nascent HDL remodeling was also performed using recombinant mouse PLTP and yielded near identical results (Figure 2B). The size difference between sn-HDL and apoA-I was also observed on gradient GGE gels, which were run to equilibrium (3000 volt/h; Figure II in the online-only Data Supplement). Similar results were obtained when apoA-I-containing species were analyzed by Western blotting (Figure III in the online-only Data Supplement).

**Cholesterol Efflux Capacity of PLTP-Modified Nascent HDL Particles**

To determine the cellular cholesterol and phospholipid efflux capacity of the sn-HDL, baby hamster kidney cells overexpressing human ABCA1 or ATP-binding cassette transporter G1 (ABCG1) were used. Cells were labeled with 0.2 μCi/μL 125I cholesterol or 1 μCi/mL 3H choline chloride for 48 hours, then incubated for 24 hours with cell medium containing sn-HDL or with apoA-I in medium with or without PLTP. Efflux of cellular 3H cholesterol or [3H]phospholipid to medium was expressed as the percentage of total radioactivity in media and cells.

The sn-HDL particles promoted cellular cholesterol or phospholipid efflux in an ABCA1-dependent manner, similar to apoA-I in the presence of PLTP. ApoA-I had a greater efflux capacity in the presence of PLTP than in the absence of PLTP. PLTP alone did not promote cholesterol or phospholipid efflux (Figure 3A and 3C). The sn-HDL served as a 15-fold better substrate for ABCA1-dependent efflux compared with ABCG1-dependent efflux. Nevertheless, the sn-HDL had a 3-fold greater capacity to promote ABCG1-dependent cholesterol efflux in the presence of PLTP than apoA-I (Figure 3B). Neither sn-HDL nor apoA-I in the presence or absence of PLTP promoted ABCG1-dependent phospholipid efflux, whereas HDL promoted ABCG1-dependent phospholipid efflux as expected (Figure 3D).

**Plasma Turnover of PLTP-Modified Nascent HDL Particles in Human apoA-I Transgenic Mice**

Previous reports have indicated that different sized nascent HDLs exhibit different plasma clearance rates. We next examined whether plasma clearance of sn-HDL in vivo differs from apoA-I. A tracer of 1.4 μg 125I-sn-HDL (≈1×10^6 cpm in 100 μL) in cell medium was injected via tail vein into a C57BL/6-Tg (APOA1) 1Rub/J mouse. This strain of mice was used because HDL in these animals contains predominantly human A-I and because apoA-I from human and mouse interacts differently with HDL. An equivalent amount of 125I-apoA-I, preincubated with the same amount of PLTP-containing medium that was used to generate sn-HDL, was studied for comparison. At the selected intervals after tracer injection (3 minutes to 4 hours), plasma samples were collected and 125I determined. At 4 hours after tracer injection, the mice were humanely killed, and livers and kidneys were collected and radioassayed.

It is evident from the plasma clearance curves that clearance of 125I-sn-HDL was significantly slower (≈3-fold) compared with lipid-free apoA-I (Figure 4A). The times taken to clear 50% of the injected doses of sn-HDL and apoA-I were 0.97±0.014 and 0.35±0.017 hours (P<0.0001), respectively. It is also evident that clearance of both ligands is biphasic with more rapid clearance of apoA-I than sn-HDL during both phases. Clearance of HDL apoA-I has been best described using a 2 pool model with a rapid initial phase being associated with an equilibration of ligand between the vascular
and extravascular pools. An accurate determination of the fractional clearance rates of the 2 ligands will require a more extensive data set than shown in Figure 4.

Analysis of plasma samples by nondenaturing GGE demonstrated that injected $^{125}$I-sn-HDL was rapidly incorporated into larger HDL-sized particles and more rapidly than $^{125}$I-apoA-I (Figure 4B). Interestingly, incorporated $^{125}$I-sn-HDL protein appeared to be less rapidly cleared from plasma than HDL-associated $^{125}$I-apoA-I. After 4 hours of tracer injection, significant amounts of $^{125}$I-sn-HDL protein remained associated with HDL-sized particles, whereas the majority of $^{125}$I-apoA-I had been cleared from such particles. A more efficient incorporation of sn-HDL into HDL particles that have a slower clearance rate may contribute to its reduced plasma clearance compared with apoA-I. In contrast, $^{125}$I-apoA-I rapidly disappears from the unlipidated and the HDL-sized fraction. This suggests that the $^{125}$I-apoA-I association with HDL may be less firmly bound than in the case of $^{125}$I-sn-HDL and might therefore contribute to its rapid clearance from plasma.

Because lipid-free and lipid-poor apoA-I has been shown to clear more rapidly from plasma than HDL, particularly through the kidney, the efficient association of sn-HDL with HDL in the plasma might therefore reduce its clearance rate as a result of reduced clearance by the kidney. To identify the possible tissue sites of clearance of apoA-I, livers and kidneys were removed from recipient mice 4 hours after tracer injection and radiolabel content was determined as percentage of injected tracer. As shown in Figure IVA and IVB in the online-only Data Supplement, $^{125}$I accumulation, in the kidney but not in the liver, is greater for $^{125}$I-apoA-I than $^{125}$I-sn-HDL despite a lower plasma concentration of $^{125}$I-apoA-I. This suggests that reduced clearance of $^{125}$I-sn-HDL in the kidney may contribute to its slower rate of clearance compared with apoA-I. To substantiate this possibility, additional studies using a nondegradable-modified apoA-I are required to quantify rates of tissue uptake of these ligands.

### Remodeling of PLTP-Modified Nascent HDL Particles In Vitro

Based on the data presented in Figure 4B, sn-HDL seems to associate with circulating HDL in vivo. To examine the ability of sn-HDL to incorporate into HDL in vitro, 50 ng in 10 μL of cell medium containing $^{125}$I-sn-HDL or $^{125}$I-apoA-I, pretreated with equal volume of PLTP (15.4 pmole/μL/h), was incubated with 15 μg of unlabeled HDL₂b or HDL₃ for indicated times at 37°C (Figure 5A and 5B). The reaction was analyzed by nondenaturing GGE and visualized by autoradiography.

The $^{125}$I-sn-HDL was rapidly associated with HDL₂b or HDL₃, as indicated by the migration of the vast majority of the radiolabel at a size corresponding to HDL after 5-minute incubation. The association of $^{125}$I-sn-HDL to mature HDL particles seemed to be more complete and less reversible with time compared with $^{125}$I-apoA-I. After 4-hour incubation, the majority of the $^{125}$I-sn-HDL remained associated with HDL₂b or HDL₃ (Figure 5A and 5B), whereas almost half of the apoA-I was dissociated from HDL₂b (Figure 5A). In the case

![Figure 4. Plasma turnover of phospholipid transfer protein (PLTP) modified nascent high-density lipoprotein (HDL) particles in human apolipoprotein A-I (apoA-I) transgenic mice. The tracer of $^{125}$I-sn-HDL or $^{125}$I-apoA-I (1.4 μg, $1 \times 10^6$ cpm in 100 μL) pre-incubated with PLTP was injected via tail vein into C57BL/6-Tg (APOA1) 1Rub/J mice (n=4). A, At the selected intervals after tracer injection (3 minutes to 4 hours), an aliquot of 10 μL plasma was radioassayed. B, An aliquot of 20 μL plasma was analyzed by nondenaturing gradient gel electrophoresis (4–20% acrylamide) and visualized by autoradiography. sn-HDL indicates small nascent HDL.](http://atvb.ahajournals.org/)

![Figure 3. Cholesterol and phospholipid efflux capacity of phospholipid transfer protein (PLTP) modified nascent high-density lipoprotein (HDL) particles. Baby hamster kidney (BHK) cells over-expressing human ATP-binding cassette transporter A1 (ABCA1; A, C) or ATP-binding cassette transporter G1 (ABCG1; B, D) were used to determine cellular cholesterol (A, B) and phospholipid (C, D) efflux to small nascent HDL (sn-HDL). Cells were labeled with 0.2 μCi/mL [3H]cholesterol or 1 μCi/ml [3H]phospholipid for 48 hours, then incubated for 24 hours at 37°C with 5 μg/mL sn-HDL or with 5 μg/mL apolipoprotein A-I (apoA-I) in the presence or absence of PLTP (activity=15.4 pmole/μL/h), PLTP alone (15.4 pmole/μL/h), or 20 μg/mL HDL₃. Efflux of cellular [3H]cholesterol or [3H]phospholipid to medium was expressed as the percentage of total radioactivity in the medium and cells together. ABCA1- and ABCG1-specific values were calculated as the difference between the efflux values in ABCA1 cells or ABCG1 cells and BHK control cells. Values shown were the means±SEM of triplicate determinations. Statistical analysis was done using unpaired, 2-tailed t tests. **P<0.01; ***P<0.001. ns indicates no significant difference.](http://atvb.ahajournals.org/)
of HDL₃, association of ¹²⁵I-apoA-I was markedly less than for ¹²⁵I-sn-HDL, and majority of ¹²⁵I-apoA-I remained non-associated with HDL (Figure 5B). These results indicated that compared with lipid-free apoA-I, the apoA-I of sn-HDL was either more readily incorporated into HDL or more effectively exchanged with apoA-I in HDL.

Discussion

PLTP plays a key role in HDL remodeling and function during physiological and pathophysiological conditions. Whether PLTP modulates the process of nascent HDL formation remains to be clarified. Because liver is the primary site for HDL biogenesis and a major source of PLTP expression, we investigated the effects of PLTP overexpression on nascent HDL formation and nascent HDL remodeling in primary mouse hepatocytes. We observed that hepatocyte overexpression of PLTP markedly affected the amount and size of lipoprotein- and apolipoprotein A-I (apoA-I) pretreated with equal volume of COS-7 culture medium containing PLTP (activity=15.4 pmole/µL/h) was incubated with 15 µg of HDL₃ (A) or HDL₄ (B) for indicated time period at 37°C. After incubation, the samples were applied to a nondenaturing gradient gel electrophoresis (4-20% acrylamide) and visualized by autoradiography.

remodeling nascent HDL particles.21 Our results demonstrated that PLTP is capable of remodeling nascent HDL particles from control hepatocytes with PLTP in vitro. PLTP in cell-conditioned medium obtained from overexpressing COS cells and recombinant PLTP yielded similar results. As shown in Figure 2, PLTP was able to modulate nascent HDL particles in vitro in a dose-dependent manner, ultimately generating particles that were <7.1 nm, but larger than apoA-I, from larger nascent HDLs. PLTP has been reported to release lipid-poor apoA-I from mature HDL particles during HDL remodeling process.21 Our results demonstrated that PLTP is capable of releasing small apoA-I species (sn-HDL) from nascent HDLs.

To examine whether PLTP modulated an early step in nascent HDL formation, namely cellular cholesterol efflux, baboon hamster kidney cells overexpressing human ABCA1 or ABCG1 were examined. PLTP did promote apoA-I-induced cholesterol efflux in an ABCA1-dependent manner (Figure 3A). These results demonstrated that PLTP is capable of enhancing apoA-I lipidation by ABCA1. This is in line with previous studies that macrophage foam cells from PLTP-deficient mice released less cholesterol to lipid-free apoA-I and to HDL than did the corresponding WT foam cells, suggesting the involvement of PLTP in cellular cholesterol efflux. It has also been reported that PLTP interaction with ABCA1 stabilizes the transporter, suggesting the significance of PLTP in ABCA1-mediated cholesterol efflux.27,28 It is possible that PLTP may function through its ability to transfer phospholipids and this notion is in line with our results showing that PLTP does promote the efflux of phospholipids to apoA-I, in addition to cholesterol efflux.

To examine the possible physiological effects of PLTP modification on nascent HDL function, we determined the efflux capacity of sn-HDL that is generated by the action of PLTP on nascent HDLs. As shown in Figure 3A, these particles promoted cellular cholesterol efflux in an ABCA1-dependent manner to similar extent as apoA-I. The cholesterol efflux induced by PLTP-modified particles was more ABCA1 dependent than ABCG1 dependent; however, sn-HDL had a
greater capacity to promote ABCG1-dependent cholesterol efflux than apoA-I (Figure 3B). ABCA1 was recognized as the principal molecule involved in cholesterol efflux to apoA-I from macrophage foam cells. In addition to ABCA1, another ATP-binding cassette transporter, ABCG1, has been shown to contribute to cholesterol efflux to HDL from macrophages. A synergistic relationship between ABCA1 and ABCG1 in promoting cholesterol efflux has been proposed. The nascent HDL particles generated through ABCA1 action were shown to function as efficient acceptors for ABCG1-mediated cholesterol efflux. In the present study, we demonstrated that PLTP-modified nascent HDL particles had a greater capacity to promote ABCG1-dependent cholesterol efflux than apoA-I, suggesting a role of PLTP in the synergistic effect of ABCA1 and ABCG1 in promoting cellular cholesterol efflux. The functional properties of sn-HDL in mediating cholesterol efflux through the different cholesterol transporters are consistent with a minimal but significant lipidation of apoA-I in sn-HDL. Together with increased size of sn-HDL and its altered capacity to associate with HDL, these results strongly support a lipidated state of apoA-I in sn-HDL.

To study the possible metabolic fate of these small PLTP-generated species in vivo, we injected 151-labeled sn-HDL into human apoA-I transgenic mice to assess the plasma turnover rate of these small particles in comparison with lipid-free apoA-I. As shown in Figure 4, in the presence of PLTP, the plasma clearance of sn-HDL was significantly slower compared with that of apoA-I. We also demonstrated that these small particles were rapidly remodeled to mature HDL-sized particles both in vivo and in plasma ex vivo, likely by fusion with preexisting HDL (Figures 4 and 5). Such remodeling of sn-HDL occurred more effectively than the remodeling of lipid-free apoA-I. Remodeling of sn-HDL resulted in particles that appeared to be less rapidly cleared in vivo than lipid-free apoA-I or particles generated from lipid-free apoA-I. It is possible that the remodeling observed involved apoA-I exchange between sn-HDL and preexisting HDL rather than a net transfer of sn-HDL onto plasma HDL. Perhaps more likely, given the relatively small size and predicted low lipid content of sn-HDL, remodeling involved the displacement of apoA-I from HDL by sn-HDL. It is likely that the structure and composition of nascent HDL particles, as well as their interaction with plasma factors, including PLTP, have a significant impact on the extent to which apoA-I is liberated from HDL. Overall, the results do provide clear evidence that sn-HDL is subject to increased remodeling and decreased plasma turnover compared with lipid-poor apoA-I. It is generally accepted that nascent HDLs acquire unesterified cholesterol and associate with lecithin:cholesterol acyltransferase, leading to cholesterol esterification and their conversion to mature HDL. Our studies provide evidence for another mechanism by which nascent HDLs can enter the mature HDL pool that is enhanced by PLTP-mediated particle remodeling.

Our findings suggest that PLTP exerts significant effects on apoA-I lipidation and nascent HDL biogenesis in hepatocytes by promoting ABCA1-dependent cholesterol efflux and the remodeling of nascent HDL particles.

Acknowledgments
We thank Xuebing Wang and Victoria Neofsiinger for excellent technical support. AdPLTP, an adenosival vector encoding mouse phospholipid transfer protein, was generously provided by Dr J. L. Goldstein. The ATP-binding cassette transporter A1 (ABCA1) antibody was generously provided by Dr M. R. Hayden. Baby hamster kidney cell expressing human ABCA1 or human N-terminal FLAG-tagged ABCG1 were a generous gift from Dr J. F. Oram.

Sources of Funding
This work was supported by National Institutes of Health Program Project Grant (PO1HL086670) to D. R. van der Westhuyzen and VA CSR&D Merit Review Award (1101CX000773) to N. R. Webb.

Disclosures
None.

References
1916 Arterioscler Thromb Vasc Biol September 2014


33. Vaughan AM, Oram JF, ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL. *J Lipid Res.* 2006;47:2433–2443.


**Significance**

Plasma high-density lipoprotein (HDL) cholesterol is an important negative risk factor for coronary heart disease. A key cardioprotective function of HDL is the delivery of cholesterol from tissues and plasma to the liver for secretion via the reverse cholesterol transport pathway. The levels and composition of HDL subclasses in plasma are regulated by many factors, including phospholipid transfer protein. Phospholipid transfer protein, which binds phospholipids and facilitates their transfer between lipoproteins in plasma, plays a key role in HDL remodeling, but its influence on nascent HDL formation is not known. The effect of phospholipid transfer protein on apolipoprotein A-I lipidation and nascent HDL biogenesis by promoting ATP-binding cassette transporter A1–dependent cholesterol efflux and the remodeling of nascent HDL particles.
Impact of Phospholipid Transfer Protein on Nascent High-Density Lipoprotein Formation and Remodeling

Ailing Ji, Joanne M. Wroblewski, Nancy R. Webb and Deneys R. van der Westhuyzen

Arterioscler Thromb Vasc Biol. 2014;34:1910-1916; originally published online July 24, 2014;
doi: 10.1161/ATVBAHA.114.303533
Arteriosclerosis, Thrombosis, and Vascular Biology is published by the American Heart Association, 7272
Greenville Avenue, Dallas, TX 75231
Copyright © 2014 American Heart Association, Inc. All rights reserved.
Print ISSN: 1079-5642. Online ISSN: 1524-4636

The online version of this article, along with updated information and services, is located on the
World Wide Web at:
http://atvb.ahajournals.org/content/34/9/1910

Data Supplement (unedited) at:
http://atvb.ahajournals.org/content/suppl/2014/07/24/ATVBAHA.114.303533.DC1

Permissions: Requests for permissions to reproduce figures, tables, or portions of articles originally published in Arteriosclerosis, Thrombosis, and Vascular Biology can be obtained via RightsLink, a service of the Copyright Clearance Center, not the Editorial Office. Once the online version of the published article for which permission is being requested is located, click Request Permissions in the middle column of the Web page under Services. Further information about this process is available in the Permissions and Rights Question and Answer document.

Reprints: Information about reprints can be found online at:
http://www.lww.com/reprints

Subscriptions: Information about subscribing to Arteriosclerosis, Thrombosis, and Vascular Biology is online at:
http://atvb.ahajournals.org//subscriptions/
Supplemental Figure I: Effect of PLTP on ABCA1 expression in hepatocytes

Primary hepatocytes were harvested 24 h after administration of control AdNull or AdPLTP (3 x 10^{10} particles) to mice. After overnight culture, some of the cells were treated with 5 μM T0901317 in Williams' Medium E containing 0.2% fatty acid-free BSA for 8 h and then incubated for 16 h with lipid-free human apoA-I (10 μg/ml) in medium containing 0.2% fatty acid-free BSA. ABCA1 mRNA expression in hepatocytes was determined by real time PCR (Supplemental Fig. 1A), and ABCA1 protein expression in hepatocytes treated with 5 μM T0901317 was determined by Western blot analysis of 10 μg cell protein analyzed on the same gel (Supplemental Fig. 1B).
Supplemental Figure II: PLTP remodels nascent HDL particles *in vitro*
Nascent HDL particles were generated by incubating primary hepatocytes with 5 μg/ml human $^{125}$I-apoA-I for 24 h. An aliquot (80 ng in 16 μl) of $^{125}$I-apoA-I lipidated particles was incubated with 8 μl of COS-7 culture medium containing PLTP (activity = 15.4 pmole/μl/h) for 16 h at 37°C. The samples were electrophoresed on non-denaturing GGE (5–30% acrylamide) for 3000 volt.h, and visualized by autoradiography.
Supplemental Figure III: PLTP remodeling of nascent HDL particles \textit{in vitro}

Nascent HDL particles were generated by incubating primary hepatocytes with 5 μg/ml human apoA-I for 24 h. An aliquot (75 ng in 15 μl) of apoA-I lipidated particles was incubated with 15 μl of COS-7 culture medium containing PLTP (activity = 15.4 pmole/μl/h) for 16 h at 37°C. The samples were analyzed by non-denaturing GGE (4–20% acrylamide), and subsequent immunoblot analysis for human apoA-I.
Supplemental Figure IV: Tissue uptake of PLTP modified nascent HDL particles in human apoA-I transgenic mice

The tracer of $^{125}$I-sn-HDL or $^{125}$I-apoA-I (1.4 μg, approx. 1 x 10^6 cpm in 100 μl) pre-incubated with PLTP was injected via tail vein into C57BL/6-Tg (APOA1) 1Rub/J mice (n = 4), the same mice as described in Fig. 4. At 4 h after tracer injection, mice were humanely killed, and livers and kidneys were collected and radioassayed. Statistical analysis was done using unpaired, two-tailed t-tests. *: P < 0.05. ns: no significant difference.
Materials and methods

Mice

C57BL/6J mice and C57BL/6-Tg (APOA1) 1Rub/J mice were purchased from the Jackson Laboratory (Bar Harbor, Maine, USA) and used at 8-12 weeks of age. Mice were maintained on a 14-h light/10-h dark cycle and received standard mouse chow and water ad libidum. Animal procedures were performed in accordance with the Public Health Service (PHS) Policy on Humane Care and Use of Laboratory Animals and with the approval of the Lexington Veterans Affairs Medical Center Institutional Animal Care and Use Committee.

HDL preparation

HDL ($\rho = 1.063–1.21$ g/ml) was isolated from fresh plasma from healthy volunteers by density gradient ultracentrifugation as described previously. All subjects gave written informed consent and the protocol was approved by the Institutional Review Board of the University of Kentucky. Human HDL was sub-fractionated by density gradient fractionation to obtain HDL$_{2b}$ ($\rho = 1.09–1.11$ g/ml), and HDL$_3$ ($\rho = 1.13–1.18$ g/ml). All isolated fractions were dialyzed against 150 mM NaCl and 0.01% EDTA, sterile filtered, and stored under nitrogen gas at 4°C. Protein concentrations were determined by the method of Lowry et al.

Primary hepatocytes culture

PLTP was expressed in livers of mice using adenoviral vector-mediated gene transfer. AdPLTP, an adenoviral vector encoding mouse phospholipid transfer protein, was generously provided by Dr. J. L. Goldstein. Primary hepatocytes were isolated from C57BL/6 mice 24 h after infusion of AdNull or AdPLTP. A two-step perfusion method was utilized for isolation of hepatocytes. Briefly, the liver was first perfused with Ca$^{2+}$/Mg$^{2+}$-free HBSS containing 10 mM glucose, 10 mM HEPES and 0.3 mM EDTA and then with HBSS containing 0.05% collagenase type IV (cat no C5138, Sigma), 1.3 mM CaCl$_2$, 0.5 mM MgCl$_2$, 10 mM glucose and 10 mM HEPES. Hepatocytes were washed by repeated low speed centrifugation (50 g for 2 min), and cell viability assessed by trypan blue exclusion was 94% ± 0.7 from six preparations. Cells were plated onto 12-well plates pre-coated with rat tail collagen (cat no 354236, BD Biosciences, Bedford, MA) at a density of $2 \times 10^5$ cells/per well and cultured at 37°C under 5% CO$_2$ in Williams’ Medium E (GIBCO) containing 10% fetal bovine serum (GIBCO), 2% penicillin-streptomycin, 1% sodium pyruvate, 1% L-glutamine and 1% insulin-transferrin-selenium (GIBCO).

Nascent HDL formation

Hepatocytes were isolated from C57BL/6 mice 24 h after infusion of AdNull or AdPLTP. After an overnight culture, cells were treated with the Liver X Receptor (LXR) agonist T0901317 (5 μM; cat no 71810, Cayman Chemical) in Williams’ Medium E containing
0.2% fatty acid-free BSA for 8 h to induce the expression of ABCA1, and then incubated with 10 µg/ml lipid-free human apoA-I (cat no A95120H, Meridian Life Science, Memphis, TN) in medium containing 0.2% fatty acid-free BSA for 16 h. The apoA-I was assessed by mass spectrometry to be phospholipid free. For some studies, apoA-I was labeled using the iodine monochloride method. The specific activity of ¹²⁵I-apoA-I ranged from 1000 to 1200 cpm/ng protein. Cell culture supernatants were harvested for analysis by non-denaturing gradient gel electrophoresis (GGE) followed by Western blotting or autoradiography.

**PLTP remodeling of nascent HDL particles in vitro**

Nascent HDL particles were generated by incubating primary hepatocytes with 5 µg/ml human ¹²⁵I-apoA-I for 24 h. Conditioned medium from COS-7 cells incubated with AdPLTP was used to generate PLTP-containing medium, analogous to the method previously described for endothelial lipase. Briefly, COS-7 cells were infected with AdPLTP at 3000 particles per cell in DMEM containing 0.2% fatty acid-free BSA for 48 h, culture medium was collected and centrifuged to remove cell debris. PLTP activity in cell culture medium was assessed by the Roar PLTP Activity Assay Kit (cat no P7700, Roar Biomedical, Inc. New York, NY). In this assay, incubation of fluorescent-labeled liposomes and unlabeled acceptors with 5 µl of conditioned medium resulted in the PLTP-mediated transfer of fluorescent phospholipids, which were present in a self-quenched state when associated with the donor. The transfer was determined by the increase in fluorescence intensity as the fluorescent lipid was removed from the donor and transferred to the acceptor. The PLTP activity was expressed as the increase in fluorescence per hour. Samples were assayed at PLTP concentrations within the linear response range of the assay. PLTP activity in COS-7 cell-conditioned medium was 15.4 ± 1.1 pmole/µl/h. By comparison, the PLTP activity of mouse plasma was determined to be 60.5 ± 1.2 pmole/µl/h.

For in vitro modification of nascent HDL particles by PLTP, an aliquot (80 ng in 16 µl) of the ¹²⁵I-apoA-I labeled nascent HDL particles was incubated with the indicated amounts of COS-7 culture medium containing PLTP for 16 h at 37°C. For some studies, an aliquot (80 ng in 16 µl) of ¹²⁵I-apoA-I labeled nascent HDL particles was incubated with the indicated amount of recombinant mouse PLTP (cat no 4918-PL-025, R & D Systems, Minneapolis, MN) for 16 h at 37°C. The samples were transferred to ice, immediately applied to a non-denaturing GGE (4–20% acrylamide), and visualized by autoradiography.

**Gel electrophoresis and Western blot analysis**

Total cell proteins (10 µg) were separated by 4-20% SDS-PAGE and transferred to PVDF membranes and immunoblotted with anti-human/mouse ABCA1 (1:750) (from Dr. M.R. Hayden) or with anti-mouse PLTP (1:500) (cat no P7722, Cardiovascular Targets, Inc. New York, NY). ApoA-I lipidation and nascent HDL particle formation was analyzed by non-denaturing GGE. Aliquots of primary hepatocyte culture medium were electrophoresed on 4-20% non-denaturing GGE for 3.5 h at 200 V, 4°C and transferred
to PVDF membranes (40 min at 100 V, 4°C) for subsequent Western blotting with anti-
human apoA-I (cat no 178463, Calbiochem) or autoradiography. For some studies, al-
liquots of hepatocyte culture medium were electrophoresed on 5-30% non-denaturing
GGE for 3000 volt.h, and visualized by autoradiography. Immunoblots were visualized
by the Amersham™ ECL™ Western Blotting Detection Reagents (cat no RPN2106, GE
Healthcare, Pittsburgh, PA). A protein standard mix containing 7.1, 8.2, 10.4, 12.2, and
17.0 nm diameter proteins was obtained from GE Healthcare (HMW Calibration Kit for
Native Electrophoresis, cat no 17-0445-01, Pittsburgh, PA).

**Real time PCR**

Total RNA was isolated from primary hepatocytes using TRI Reagent (cat no TR118,
Molecular Research Center) according to the manufacturer’s protocol. RNA samples
were treated with DNase I (cat no 04716728001, Roche) for 30 min at 37°C and then
purified with the RNeasy Mini Kit (cat no 74106, QIAGEN). RNA (2 µg) was reverse
transcribed into cDNA using High Capacity cDNA Reverse Transcription Kit (cat no
4368813, Applied Biosystems). After 4-fold dilution, 5 µl was used as a template for
real-time PCR. Amplification was done for 40 cycles using Power SYBR Green PCR
Master Mix (cat no 4367659, Applied Biosystems). Quantification was performed using
the standard curve method and normalizing to GAPDH. The primers used for ABCA1
and PLTP are as follows: mABCA1, NM_013454: forward 5′- AGCCAGAAGGGAGTGTCAGA-
3′, reverse 5′-CATGCCATCTCGGTAAACCT-3′, 102bp; mPLTP, NM_011125: forward 5′-
TGGGACGGTGTTGCTCAA-3′, reverse 5′- CCCACGAGATCATCCACAGA-3′, 69bp.

**Cholesterol and phospholipid efflux measurement**

Cellular cholesterol and phospholipid efflux were assessed using Baby hamster kid-
ny (BHK) cells as described. Briefly, BHK cells expressing human ABCA1 or human N-
terminal FLAG-tagged ABCG1 and control cells transfected with empty vector were a
generous gift from Dr. J. F. Oram. The transfected cell lines were generated using
the mifepristone-inducible GeneSwitch system. Cells were grown in DMEM
supplemented with 10% FBS, 1% penicillin and streptomycin, as well as 2 mM
glutamine. Cells (~70% confluent) in 12-well plates were labeled with 0.2 µCi/ml
[^3]H]cholesterol (35-50 Ci/mmol, cat no NET139001MC, PerkinElmer) or 1 µCi/ml
[^3]H]choline chloride (cat no TRK, Amersham Biosciences, Amersham, UK) in complete
DMEM medium for 48 h, washed twice with PBS containing 1 mg/ml BSA (PBS-BSA),
and equilibrated in DMEM containing 0.2% fatty acid-free BSA (DMEM-BSA) for 16 h.
Cellular ABCA1 and ABCG1 expression was induced by incubating with 10 nM
mifepristone (cat no H11001, Life Technologies) in DMEM-BSA during the 16 h
equilibration period. Following two additional washes with PBS-BSA, cells were
incubated for 24 h at 37°C in DMEM-BSA with nascent HDL particles remodeled by
PLTP in vitro, human apoA-I in the presence or absence of PLTP, PLTP alone, or
human HDL₃, as indicated. Following incubation, the medium was collected and
centrifuged to remove detached cells. Adherent cells were washed at 4°C twice with
PBS-BSA and twice with PBS. For cholesterol efflux, radioactivity in the medium was
measured directly in a Packard liquid scintillation counter. Cellular lipid was extracted with hexane/isopropyl alcohol (3:2 v/v) for 1 h at room temperature and counted for radioactivity. For phospholipid efflux, medium and cellular choline-labeled phospholipids were extracted in chloroform/methanol (2:1 v/v) and assayed for $^3$H radioactivity. Efflux of cellular $[^3]$H cholesterol and $[^3]$H phospholipid to medium was expressed as the percentage of total radioactivity in media and cells. ABCA1- and ABCG1-specific values were calculated as the difference between the efflux values in ABCA1 cells or ABCG1 cells and BHK control cells. Values shown were the mean ± SEM of triplicate determinations.

**Plasma turnover of PLTP modified nascent HDL particles in human apoA-I transgenic mice**

The PLTP modified small nascent HDL particles (sn-HDL) were generated by incubating $^{125}$I-apoA-I nascent HDL particles with equal volume of COS-7 culture medium containing PLTP for 16 h at 37°C as described above. The sn-HDL containing medium was concentrated using Amicon Ultra 10K concentrators and a tracer amount containing 1.4 µg sn-HDL (approx. 1 x $10^6$ cpm in 100 µl) was injected via tail vein into C57BL/6-Tg (APOA1) 1Rub/J mice ($n = 4$). An equivalent amount of $^{125}$I-apoA-I pre-incubated with PLTP was injected for comparison ($n = 4$). At the selected intervals after tracer injection, plasma was collected from the retro-orbital sinus, radioassayed, and analyzed by non-denaturing GGE (4–20% acrylamide). At 4 h after tracer injection, the mice were humanely killed, and livers and kidneys were collected and radioassayed.

**Remodeling of PLTP modified nascent HDL particles in vitro**

PLTP modified nascent HDL particles were obtained by incubating $^{125}$I-apoA-I nascent HDL particles (50 ng in 10 µl) with equal volume of COS-7 culture medium containing PLTP (15.4 pmole/µl/h) for 16 h at 37°C. The sample was then mixed with 15 µg of HDL$_{2b}$ or HDL$_3$ and incubated for indicated time period at 37°C. An equivalent amount of $^{125}$I-apoA-I pre-incubated with PLTP containing COS-7 culture medium was mixed with the same amount of HDL$_{2b}$ or HDL$_3$ and incubated for indicated time period for comparison. After incubation, the samples were transferred to ice, and then immediately applied to a non-denaturing GGE (4–20% acrylamide) and visualized by autoradiography.

**Statistical Analysis**

Results are expressed as the mean ± SEM as indicated in the figure legends. Where not visible, error bars are contained within the symbols for results with $n \geq 3$. Best-fit two phase curves were generated using GraphPad Prism 4 software. Statistical analysis was done using unpaired, two-tailed $t$-tests. $P < 0.05$ was considered statistically significant.


