Objective—Plasma phospholipid transfer protein (PLTP) is involved in the metabolism of HDL and apolipoprotein B (apoB)-containing lipoproteins. Atherosclerosis susceptibility is decreased in mice with PLTP deficiency that is associated with decreased liver production of apoB-containing lipoproteins and increase in their antioxidant. To investigate additionally the effect of PLTP on the development of atherosclerosis, we overexpressed PLTP in mice.

Methods and Results—PLTP was overexpressed in apoE knockout mice using an adenovirus-associated virus (AAV)-mediated system. Plasma PLTP activity was 1.3- to 2-fold higher in mice injected with AAV-PLTP than in mice injected with control AAV-GFP, and PLTP levels were sustained during the experiment period (4 months). We show that 2-fold increased PLTP activity results in (1) a decrease in HDL cholesterol, HDL phospholipid, and apoAI levels; (2) a decrease in vitamin E contents in total plasma and in individual lipoprotein fractions; (3) an increase in lipoprotein oxidizability as assessed by copper-induced formation of conjugated dienes; (4) an increase in autoantibodies against oxidized apoB-containing particles; and (5) an increase in atherosclerosis lesions in proximal aorta.

Conclusions—These observations indicate that elevated plasma PLTP levels constitute a novel, long-term risk factor for atherosclerosis.

Key Words: phospholipid transfer protein • apoB-containing lipoproteins • oxidation • high-density lipoprotein • atherosclerosis

Several hydrophobic compounds that are normally associated with circulating lipoproteins can exchange in the plasma compartment through the activity of lipid transfer proteins, ie, cholesteryl ester transfer protein and phospholipid transfer protein (PLTP).1 Although most net mass transfers of phospholipids in plasma can be ascribed to PLTP,2 studies in the past decade have built a complex picture of the role of PLTP in lipoprotein metabolism and vascular biology. Indeed, besides phospholipid transfer activity, PLTP can bind and transfer several other amphipathic compounds, including α-tocopherol, diacylglycerides, cerebrosides, and lipopolysaccharides.3 The apparent complexity and multiplicity of PLTP actions raise the key questions of its role in lipoprotein metabolism in vivo and the consequences in terms of atherosclerosis susceptibility.

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Genetically engineered mice, ie, one animal species with initially high plasma PLTP activity,4,5 were of considerable help in clarifying the function of PLTP in vivo. The phenotypes of either PLTP transgenic or PLTP knockout mice came in support of a major role of PLTP in the metabolism of HDL and apolipoprotein B (apoB)-containing lipoproteins, influencing the concentration, apolipoprotein content, and size of plasma lipoproteins.6–8 Recent data indicated that PLTP deficiency in mice is associated with a decrease in atherosclerosis susceptibility despite concomitant decrease in plasma HDL levels. Complementary metabolic studies revealed that at least 2 distinct molecular mechanisms would account for the reduction in atherosclerosis in PLTP-deficient animals, including the reduction in the liver production and plasma levels of potentially atherogenic apoB-containing lipoproteins9 and the rise in the antioxidative potential of apoB-containing lipoproteins attributable to the accumulation of vitamin E.10

Recent studies in humans revealed that both PLTP mass and activity tend to be abnormally elevated under physiological or pathological states that are known to be associated with increased atherogenesis susceptibility, including aging, obesity, and diabetes.11–15 bringing indirect support to a putative proatherogenic property of PLTP. However, to date, no direct link between high PLTP activity and increased atherosclerosis susceptibility has yet been demonstrated in humans. In a recent study,16 it was shown that overexpression...
of PLTP in mice heterozygous for the LDL receptor increased atherosclerotic susceptibility attributable to decreased HDL levels. In this study, we also try to address the same question using a different animal model. The vitamin E content of apoB-containing lipoproteins, their susceptibility to undergo oxidative transformation, as well as the development of atherosclerotic lesions were monitored in apoE knockout (apoE0) mice that were injected with adenovirus-associated virus (AAV) PLTP. Overall, PLTP overexpression reversed the beneficial phenotype of PLTP deficiency, indicating that PLTP is a novel risk factor for atherosclerosis.

Methods

Mice
ApoE0 mice (6-week-old females) in the C57BL/6 background were purchased from Jackson Laboratory (Bar Harbor, Maine). The mice were fed a chow diet (Purina Rodent Chow, No. 5001, Research Diets Inc). All experiments involving animals were conducted under the approval of SUNY Downstate Medical Center IACUC.

PLTP Overexpression
The recombinant AAV vector was made by inserting the mouse PLTP cDNA between the albumin enhancer/promoter (2.3-kb fragment) and bovine growth hormone polyA site. The entire liver-specific expression cassette was then flanked by the AAV2 inverted terminal repeats in the vector plasmid.18 The AAV2 viral particles were packaged and purified as reported previously using the adenovirus-free, triple-plasmid transfection method.19 The AAV2 vector was prepared in 293 cells as previously described,18 and titer (viral genome particles per milliliter) were determined by viral DNA dot blot method. In the present study, 2 doses of AAV-PLTP or AAV-GFP (control vector) were injected into apoE0 mice (5 x 1011 particles per 25 g) through the leg vein. PLTP activity was measured before injection, and it was monitored along a 4-month period after injection.

PLTP Activity Assay
PLTP activity was measured with a commercially available fluorescent assay kit that includes donor and acceptor particles (Cardiovascular Target, Inc). Incubation of fluorescent-labeled liposomes and unlabeled acceptors with 3 μL of mouse plasma results in the PLTP-mediated transfer of fluorescent phospholipids, which are present in a self-quenched state when associated with the donor. The transfer is determined by the increase in fluorescence intensity as the fluorescent lipid is removed from the donor and transferred to the acceptor. The linear range of plasma PLTP activity in this assay was between 2 and 10 μL of mouse or human plasma. The interassay coefficient of variation of the plasma PLTP activity was 2.7 ± 0.2%. The detailed procedure will be published elsewhere.

Lipid and Lipoprotein Analyses
Total lipoproteins and lipoprotein fractions (total lipoproteins, 1.006 to 1.21 g/mL; non-HDL, 1.006 to 1.063 g/mL; and HDL, 1.063< d <1.21 g/mL) were obtained by ultracentrifugation, and cholesterol and phospholipid contents in each lipoprotein fractions were measured as reported before.20 Lipoprotein profiles were obtained by fast protein liquid chromatography using 2 Sepharose 6B columns.7 The fraction size was 0.7 mL per fraction. VLDL was contained in fractions 20 to 23, LDL was contained in fractions 24 to 26, and HDL was contained in fractions 30 to 34. Total lipoprotein fractions were used for apolipoprotein analysis on SDS-PAGE. Total cholesterol and phospholipids in total plasma and isolated lipoprotein fractions were assayed using commercially available enzymatic kits (Wako Pure Chemical Industries Ltd).

α-Tocopherol Quantitation in Plasma and in Isolated Lipoproteins
Lipophilic compounds were extracted from lipoprotein fractions by an ethanol/hexane solution (1:3 vol/vol), as previously described.21 The hexane fraction was evaporated under nitrogen, and it was finally recovered in methanol. α-Tocopherol was assayed by HPLC as described before.22 Briefly, samples were loaded on a Beckman Gold System equipped with a 4.6-mm×7.5-cm Beckman Ultrasphere ODS-3 microns column that was connected to a Shimadzu Fluorescence Detector (RF-10AXL), Tocil (Spiral-Couteron) was added to each sample as an internal standard before the extraction.

Conjugated Diene Formation
ApoB-containing particles (1.006< d <1.063 g/mL) from apoE0 mice were isolated by ultracentrifugation. The lipoproteins were oxidized at 37°C in the presence of copper sulfate (5 μmol/L), and the formation of conjugated dienes was monitored at 234 nm over a 5-hour period.

Measurement of Antioxidized ApoB-Containing Particle Autoantibodies
Diluted plasma was added in microwells that were coated with copper-oxidized VLDL/LDL. Bound autoantibodies were then detected with anti-mouse IgG antibodies coupled to alkaline phosphatase. Bound antibodies were finally detected in the presence of a chemiluminescent substrate.

Atherosclerosis Assays
Proximal aortic lesion area was assessed 4 months after infection of 6-week-old apoE0 mice with either AAV-PLTP or AAV-GFP. Mice were killed, and hearts and proximal aortae were removed. Several sections (10-μm-thick) that are spaced over the span of the aortic root (usually 11 to 12 total) were obtained, and we then picked sections 2 (next to the very beginning of the aortic root), 4, 6, 8, and 10 for Oil Red O staining. The mean area of lipid staining per section from 5 sections was used for atherosclerotic lesion quantitation. Because the distributions of lesion areas were not normal, the values were square root transformed. The statistical significance of the differences between the groups was estimated by Wilcoxon rank-sum test.

Results

PLTP Expression by AAV Injection in Mice
Previous studies demonstrated that PLTP deficiency in PLTP knockout (PLTP0) mice can be completely corrected by 1 injection of AAV-PLTP (5 x 1011 particles per 25 g).9 By using the same experimental approach, a sustained PLTP overexpression could be obtained in the present study by a single injection of AAV-PLTP in hyperlipidemic apoE0 mice with elevated atherosclerosis susceptibility.21 To study the dosage effect of PLTP overexpression, in this study we used 2 doses of AAV-PLTP, 5 x 1011 and 5 x 1011 particles per 25 g, respectively, because a 2-log-range linear dose-response curve of transgene expression could be achieved within these 2 doses.24 As shown in Figure 1, a significant increase in plasma PLTP activity was observed as soon as 1 month after the high-dose AAV-PLTP injection, with no effect after AAV-GFP injection. The maximal 1.3- and 2-fold rise in PLTP activity for low- and high-dose injections, respectively, was reached after 2 months, and the overexpression was maintained up to 4 months after injection.
Effect of PLTP Overexpression on Atherosclerotic Lesion

To evaluate the effect of PLTP overexpression on atherogenesis, mean atherosclerotic lesion area was determined in proximal aorta from apoE0 mice with AAV-PLTP or AAV-GFP injection. As soon as 4 months after high-dose AAV-PLTP infection, apoE0 mice overexpressing PLTP showed a marked 2-fold rise in mean lesion area compared with AAV-GFP–infected apoE0 mice (Figure 2B). No significant changes were observed in low-dose injected animals (Figure 2A). To study the mechanisms regarding the relationship between overexpression of PLTP and induction of atherogenesis, we performed the following experiments.

Effect of PLTP Overexpression on the Plasma Lipids and Lipoproteins

As indicated in Table 1, 4 months after high-dose AAV-PLTP injection in apoE0 mice, a significant reduction in HDL cholesterol and HDL phospholipids (−34% and −31%, respectively) was observed compared with AAV-GFP–injected controls. No significant difference in non-HDL cholesterol or phospholipid was observed in high-dose AAV-PLTP–injected animals. Moreover, the reduction of HDL was not observed in low-dose AAV-injected mice, indicating a dose-dependent effect. AAV-PLTP–mediated alterations in the distribution of cholesterol was confirmed by gel permeation chromatography of pooled plasma samples (data not shown), with a marked 30% reduction in HDL cholesterol levels of apoE0 mice injected with high dose of AAV-PLTP compared with apoE0 mice injected with the same dose of AAV-GFP. Electrophoresis on SDS polyacrylamide gradient gels also revealed that high-dose AAV-PLTP injection resulted in 50% reduction of apoAI content compared with that of control (data not shown). There were no significant changes in the low-dose–injected animals in either cholesterol distribution or apoAI levels. We have measured paraoxonase in these samples and have not found any significant changes. Also, we have measured phospholipid composition (phosphatidylcholine and sphingomyelin) in HDL and have not found any significant changes (data not shown).

**Table 1. Lipid Levels in Total Plasma and Lipoprotein Fractions From AAV-PLTP- and AAV-GFP–Injected ApoE0 Mice**

<table>
<thead>
<tr>
<th></th>
<th>Cholesterol, mg/dL</th>
<th>Phospholipids, mg/dL</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>AAV-GFP</td>
<td>AAV-PLTP</td>
</tr>
<tr>
<td>Low-dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>471±81</td>
<td>433±31</td>
</tr>
<tr>
<td>Non-HDL</td>
<td>425±91</td>
<td>382±55</td>
</tr>
<tr>
<td>HDL</td>
<td>46±8</td>
<td>53±6</td>
</tr>
<tr>
<td>High-dose</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Plasma</td>
<td>462±92</td>
<td>392±66</td>
</tr>
<tr>
<td>Non-HDL</td>
<td>411±102</td>
<td>355±44</td>
</tr>
<tr>
<td>HDL</td>
<td>53±7</td>
<td>35±8*</td>
</tr>
</tbody>
</table>

n=8 to 10.

*P<0.01 (AAV-GFP vs AAV-PLTP).
Effect of PLTP Overexpression on the α-Tocopherol Content and Oxidizability of Circulating Lipoproteins

Two-fold PLTP overexpression resulted in a significant reduction in the total α-tocopherol concentration in plasma from AAV-PLTP-injected mice compared with plasma of AAV-GFP-injected mice (−20%, \( P<0.01 \), Table 2). Detailed analysis of individual, ultracentrifugally isolated lipoprotein fractions indicated that PLTP overexpression actually led to significant reductions in the α-tocopherol to lipid ratio in any lipoprotein fraction, ie, VLDL, LDL, and HDL (Table 2). Beyond abnormalities of vitamin E distribution in plasma from homozygous PLTP knockout mice,10 the present findings indicate an essential effect of PLTP in influencing the vitamin E content of lipoproteins.

To determine whether the PLTP-mediated decrease in vitamin E content of atherogenic lipoproteins rendered them more susceptible to oxidative transformations, the VLDL+LDL fractions from apoE0 mice injected with AAV-PLTP or AAV-GFP were ultracentrifugally isolated, and the generation of conjugated dienes during the incubation in the presence of 5 μmol/L copper sulfate was monitored at 234 nm over a 5-hour period. As shown in Figure 3, the lag phase of conjugated dienes formation and their accumulation was considerably accelerated by 2-fold but not 1.3-fold PLTP overexpression. The lag phase for the AAV-GFP–injected group is 40±10 minutes, whereas the lag phase for the AAV-PLTP–injected group is 10±5 minutes (\( P<0.01 \)).

Autoantibodies to oxidized apoB-containing particles are known to progressively rise over time in cholesterol-fed LDL receptor knockout mice25,26; their titer correlates with the extent of atherosclerosis.25 From our previous study,10 we found that PLTP deficiency caused significant reduction of the autoantibodies in mouse plasma. To see the effect of PLTP overexpression on the levels of the autoantibodies, we measured their title and found that 2-fold but not 1.3-fold PLTP overexpression was accompanied by a 50% significant induction in the titer of the autoantibodies (Figure 4), confirming that plasma PLTP levels have a direct and dose-dependent effect on the autoantibodies, although the mechanism is still unknown.

Discussion

We have previously shown that complete PLTP deficiency provides protection against atherosclerosis in several models of hyperlipidemic mice.9 At least 2 distinct mechanisms might account for the least atherosclerosis susceptibility of PLTP0 mice, including decreased hepatic production and circulating levels of apoB-containing atherogenic lipoproteins9 as well as alterations of their antioxidation properties.10 The present study demonstrates additionally that PLTP can actually influence atherogenicity in a dose-dependent manner, with a visible deleterious effect of a significant rise in plasma PLTP activity in mice injected with AAV-PLTP. Thus, a 2-fold overexpression of PLTP in hyperlipidemic apoE0 mice was shown to result in (1) a decrease in plasma HDL cholesterol, HDL phospholipid, and apoAI levels; (2) a decrease in the vitamin E content of all plasma lipoproteins; (3) an increase in oxidizability of apoB-containing lipopro-

Table 2. α-Tocopherol Levels in Total Plasma and Lipoprotein Fractions From AAV-PLTP– and AAV-GFP–Injected ApoE0 Mice

<table>
<thead>
<tr>
<th></th>
<th>AAV-GFP (n=5)</th>
<th>AAV-PLTP (n=5)</th>
<th>( P )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Total plasma, ( \mu g/mL )</td>
<td>5.58±0.24</td>
<td>4.42±0.29</td>
<td>0.01</td>
</tr>
<tr>
<td>VLDL (( \alpha )-tocopherol/lipid, ( \mu g/mg ))</td>
<td>0.66±0.05</td>
<td>0.58±0.05</td>
<td>0.02</td>
</tr>
<tr>
<td>LDL (( \alpha )-tocopherol/lipid, ( \mu g/mg ))</td>
<td>0.47±0.05</td>
<td>0.38±0.01</td>
<td>0.02</td>
</tr>
<tr>
<td>HDL (( \alpha )-tocopherol/lipid, ( \mu g/mg ))</td>
<td>0.64±0.04</td>
<td>0.35±0.09</td>
<td>0.01</td>
</tr>
</tbody>
</table>

Figure 3. Time-course of the formation of conjugated dienes (absorbance at 234 nm) in apoB-containing lipoproteins. Pooled apoB-containing lipoproteins were isolated from apoE0 mice with low- and high-dose AAV-PLTP or AAV-GFP injection. Values correspond to mean±SD of 3 distinct experiments. A, Low dose; B, high dose.

Figure 4. Effect of PLTP overexpression on the circulating levels of antioxidant VLDL/LDL autoantibodies. The levels of oxidized VLDL/LDL autoantibodies were determined as described in Methods. Data are mean±SD of \( n=7 \) animals. A, Low dose; B, high dose.
teins; (4) an increase in autoantibodies against oxidized lipids; and (5) an increase in mean atherosclerotic lesions.

Overexpression of PLTP in wild-type mice within a short period of time (no more than 3 weeks) was achieved by adenovirus-mediated infection, which resulted in a 10- to 40-fold increase in plasma PLTP activity. Increased preβ-HDL levels and decreased α-HDL levels were observed in those mice. Observed changes were explained by an increased fractional catabolic rate of HDL and an enhanced hepatic uptake of HDL cholesterol esters. Recently, transgenic mice that overexpress human PLTP were generated. A 2.5- to 4.5-fold increase in plasma PLTP activity results in a 30% to 40% decrease of plasma levels of HDL cholesterol. It is interesting to note that both PLTP deficiency and PLTP overexpression decrease HDL levels. Although similar causes, ie, decreased lipid content of HDL, may produce the same effect in both cases, the molecular mechanisms are probably different. In the case of PLTP deficiency, the blockade of the transfers of phospholipids and cholesterol from triglyceride-rich lipoproteins into HDL may influence negatively the formation of mature HDL by decreasing their lipidation. The formation of PLTP overexpression facilitates redistribution of phospholipids and cholesterol among lipoproteins would accelerate the delipidation of HDL, because plasma PLTP enhances both the net transfer of VLDL phospholipids into HDL and the exchange of the phospholipids among lipoproteins. In both cases, this would result in increased fractional catabolic rate of HDL and enhanced hepatic uptake of HDL cholesterol esters.

In the present study, we demonstrated that PLTP overexpression increases the extent of atherosclerotic lesions in aorta of hyperlipidemic mice. In this context, the AAV-PLTP infection of apoE0 mice was particularly relevant, both in terms of duration (ie, the expression of PLTP is sustained for at least 4 months, as shown in the present study) and atherosclerosis susceptibility (ie, atherosclerotic lesions are routinely observed in hyperlipidemic apoE0 mice, as shown in the present study). These observations, which were just the opposite of those obtained from PLTP0 mice in which PLTP deficiency led to fewer atherosclerotic lesions, provide additional support of a proatherogenic property of PLTP. In view of the fact that the expression of PLTP at 1.3-fold, the normal level in mice, does not have a significant impact on either the lipid levels in the plasma, the functional behavior of the lipoproteins, or atherosclerosis, we speculate that there should be a threshold for PLTP showing its atherogenic property. Indeed, coronary artery disease patients within the highest quintile of PLTP activity revealed a 1.9-fold increase in risk for coronary artery disease compared with patients within the lowest quintile (95% CI, 1.3 to 2.8) (Blankenberg and Jiang, unpublished observations, 2003).

As expected, the 2.0-fold increase in PLTP activity in AAV-PLTP/apoE0 mouse plasma produced a significant reduction in HDL cholesterol and apoAI. Because HDL levels are negatively related to atherosclerosis in human, it is tempting to speculate that lower HDL levels in AAV-PLTP–infected apoE0 mice might contribute, at least in part, to the development of atherosclerosis. During the preparation of this manuscript, a similar study using PLTP transgenic mice with heterozygous LDL receptor deficiency indicated that PLTP overexpression caused increase of atherosclerosis attributable to decrease of HDL levels. However, it must be emphasized that PLTP deficiency with low atherogenic potential is also associated with decreased HDL levels in mouse plasma. The latter point did not come in strong support of a direct relationship between the ability of PLTP to lower plasma HDL levels and the rise in atherosclerosis susceptibility. Similarly, although decreased levels of apoB-containing lipoproteins were proven to contribute significantly to the weaker atherosclerosis susceptibility in hyperlipidemic mice with PLTP deficiency, this explanation did not hold in AAV-PLTP–infected mice, which displayed in the present study similar plasma VLDL/LDL levels as AAV-GFP–infected mice with significantly fewer atherosclerosis lesions. There may be 2 reasons to explain why PLTP overexpression in this study did not alter the VLDL/LDL and apoB levels. First, 2-fold increase of PLTP expression was still not high enough to have an effect on VLDL production. Indeed, it was reported that more than 2-fold overexpression of PLTP caused increase of VLDL secretion in mice. Second, in hepatocytes, the amount of PLTP involved in VLDL assembly is limited; thus, AAV-mediated overexpression of PLTP may only influence PLTP levels in the circulation but not inside the hepatocytes.

Besides PLTP-mediated alterations in plasma lipid parameters, abnormalities in the oxidation susceptibility of apoB-containing lipoproteins, and, as a consequence, in their atherogenic properties, constitutes a cogent explanation. Indeed, PLTP overexpression in AAV-PLTP–infected mice produced a marked and significant rise in the oxidation of apoB-containing lipoproteins as monitored ex vivo in the presence of copper sulfate. Oxidative modification of LDL is known to facilitate its uptake by macrophages, promoting the formation of foam cells, and the presence of oxidized epitopes in atherosclerotic lesions supports an important role of oxidative modification in atherogenesis. In AAV-PLTP mice, the increased oxidizability of apoB-containing lipoproteins and the increased autoantibodies to oxidized of those particles (Figures 3 and 4) were accompanied by a concomitant and significant decrease in vitamin E, one of the major antioxidative compounds in circulating lipoproteins. PLTP has been shown in vitro to facilitate the transfer of vitamin E from VLDL to HDL and from lipoproteins into tissues. It is also known that PLTP deficiency regulates vitamin E levels in lipoproteins in vivo. Although some studies came in direct support of a beneficial role of vitamin E dietary supplementation in preventing oxidative transformation of apoB-containing lipoproteins and atherosclerosis, the relationship between vitamin E and atherosclerosis is not quite clear in humans, with most studies being negative. However, it must be emphasized at this stage that the lack of detailed and systematic analysis of vitamin E distribution among circulating lipoproteins may have constituted a potential shortcoming of the human studies, and in particular determinants of the bioavailability of antioxidants in relevant sites such as the apoB-containing lipoproteins have not been clearly identified. The present study in AAV-PLTP–infected mice, together with our previous report in PLTP0 mice,
indicates that PLTP represents one such factor determining vitamin E concentration in apoB-containing lipoproteins and plasma. Our data bring new insights into a major role of PLTP in determining the antioxidant defense and atherogeneity of lipoproteins. PLTP might well account for the discordance observed between the susceptibility of LDL to oxidation ex vivo and the dietary intake of vitamin E, one hypothesis that deserves additional investigation in human populations.

Acknowledgments

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References


Increased Atherosclerotic Lesions in ApoE Mice With Plasma Phospholipid Transfer Protein Overexpression
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