# Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study

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**Abstract Plasma phospholipid transfer protein (PLTP) interacts with HDL particles and facilitates the transfer of phospholipids from triglyceride (TG)-rich lipoproteins to HDL. Overexpressing human PLTP in mice increases the susceptibility to atherosclerosis. In human plasma, high-active and low-active forms of PLTP exist. To elucidate the contribution of phospholipid transfer activity to changes in lipoprotein metabolism and atherogenesis, we developed mice expressing mutant PLTP, still able to associate with HDL but lacking phospholipid transfer activity. In mice heterozygous for the LDL receptor, effects of the mutant and normal human PLTP transgene (mutPLTP tg and PLTP tg, respectively) were compared. In PLTP tg mice, plasma PLTP activity was increased 2.9-fold, resulting in markedly reduced HDL lipid levels. In contrast, in mutPLTP tg mice, lipid levels were not different from controls. Furthermore, hepatic VLDL-TG secretion was stimulated in PLTP tg mice, but not in mutPLTP tg mice. When mice were fed a cholesterol-enriched diet, atherosclerotic lesion size in PLTP tg mice was increased more than 2-fold compared with control mice, whereas in mutPLTP tg mice, there was no change. Our findings demonstrate that PLTP transfer activity is essential for the development of atherosclerosis in PLTP transgenic mice, identifying PLTP activity as a possible target to prevent atherogenesis, independent of plasma PLTP concentration.**—Samyn, H., M. Moerland, T. van Gent, R. van Haperen, J. Metso, F. Grosveld, M. Jauhiainen, A. van Tol, and R. de Crom. **Plasma phospholipid transfer activity is essential for increased atherogenesis in PLTP transgenic mice: a mutation-inactivation study.** ***J. Lipid Res.*** **2008.** 49: **2504–2512.**

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Phospholipid transfer protein (PLTP) is a multifunctional protein secreted by various cell types into the plasma, where it associates with HDL particles. Plasma PLTP has a central role in cholesterol and lipoprotein metabolism. It mediates the transfer of phospholipids between lipoprotein particles (1, 2). In addition, PLTP in vitro is able to transfer other lipophilic substances such as diacylglycerol (3), cholesterol (4), lipopolysaccharide (5, 6), and α-tocopherol (7), an important anti-oxidant. Furthermore, plasma PLTP has been identified as an HDL conversion factor. It remodels HDL to generate large particles and small lipid-poor pre-β-HDL (8–10). In vitro, HDL conversion depends on phospholipid transfer activity of PLTP (11).

Studies using genetically modified mouse models have provided more insight into the (patho-) physiological role of PLTP in lipoprotein metabolism and the development of atherosclerosis. PLTP-deficient mice have markedly reduced HDL levels (12). These mice are more resistant to atherosclerosis development; this is partly attributable to diminished secretion and lower levels of apolipoprotein B (apoB)-containing lipoproteins and to the increase in bioavailability of vitamin E in these particles (13, 14). When overexpressing human PLTP in mice, elevation of plasma PLTP activity levels results in a dose-dependent decrease in HDL levels, despite an increased production of pre-β-HDL particles (15). Hepatic VLDL secretion rates are increased in PLTP transgenic mice (16). We previously generated different mouse lines with either ubiquitous or liver-specific overexpression of human PLTP. Regardless of the site of PLTP production, a positive correlation between plasma PLTP activity and atherosclerotic plaque size was

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Abbreviations: apoA-I, apolipoprotein A-I; AU, arbitrary unit; FPLC, fast-protein liquid chromatography; FXR, farnesoid X receptor; HFHC, high-fat, high-cholesterol; IAA, iodoacetate; LDLR, LDL receptor; mutPLTP, mutant PLTP; PLTP, phospholipid transfer protein; TC, total cholesterol; tg, transgenic; TG, triglyceride.

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observed, suggesting that systemic PLTP is the cause of the increased susceptibility to developing atherosclerosis in these mouse models (16). Exactly how elevated PLTP levels lead to increased atherosclerosis is still not fully understood.

Until now, studies regarding the relationship between PLTP and atherosclerosis in mice focused on the absence of PLTP protein in plasma (PLTP knockout mice) or on the presence of functional PLTP with phospholipid transfer activity.

However, in human plasma, as well as in plasma of mice with adenoviral overexpression of human PLTP, two forms of PLTP have been described (17, 18), which may be regulated differently (19). In addition to the catalytically active form, a low-active form with unkown function also exists. To provide more insight into the role of PLTP transfer activity and PLTP mass in vivo, we generated a new mouse model with overexpression of a mutant human PLTP protein in the liver. The mutant has previously been described in vitro by Huuskonen et al. (20). The mutation in the N-terminal lipid binding pocket of the PLTP protein interfered with its phospholipid transfer activity in vitro, but did not affect binding to HDL. Our newly developed mouse model expresses human PLTP that associates with HDL in plasma, but lacks phospholipid transfer activity.

In the present study, we investigate the importance of PLTP transfer activity in lipoprotein metabolism, pre-β-HDL formation, hepatic VLDL secretion, and the development of atherosclerosis, by comparing mice expressing human PLTP with and without transfer activity with their control littermates.

## MATERIALS AND METHODS

### Animals

The generation of transgenic mice with liver-specific expression of human PLTP has been described previously (16). In short, the complete human PLTP sequence was isolated and the genomic DNA sequence between the *BamH*I sites in exons 5 and 8 was replaced by the equivalent cDNA sequence. The albumin promoter and enhancer sequences were cloned into the first exon of the human PLTP gene, and a 6.5 kb fragment containing the 3 α-fetoprotein enhancer elements was cloned downstream from exon 16. The PLTP transgenic (PLTP tg) mice used in the present study are derived from the mouse line referred to as A3 in our previous work (16). Mutant PLTP transgenic (mutPLTP tg) mice were generated as described for PLTP transgenic mice, but a mutation was introduced in the cDNA sequence of the construct. The replacement of TGG nucleotides by CTT through PCR-based site-directed mutagenesis changed the leucine residue 196 in the N-terminal pocket of PLTP into tryptophan (L196W) (20). Transgenic mice were crossed into a C57BL/6 background for at least eight generations. Transgenic mice hemizygous for human PLTP or mutant human PLTP were crossed with LDL receptor (LDLR)-deficient mice (obtained from Jackson Laboratory) to obtain PLTP transgenic*LDLR$^{+/-}$ and mutant PLTP transgenic*LDLR$^{+/-}$, or LDLR$^{+/-}$ control littermates. For convenience, these mice are referred to as "PLTP tg" mice, "mutPLTP tg" mice, and "control" mice, respectively. All mice used in the study were kept on regular chow diet or on an atherogenic high-fat, high-cholesterol (HFHC) diet containing 40% (w/w) sucrose, 15% (w/w) cocoa butter, 1% (w/w) cholesterol, and 0.5% (w/w) cholate (diet N; Hope Farms, Woerden, The Netherlands) for 13 weeks. Only male mice were used in the study. The mice were 10–12 weeks old at the beginning of the diet studies. All animals had free access to food and water. To perform plasma analyses, blood was collected from the orbital plexus after an overnight fasting period. All animal experiments were carried out in compliance with national and institutional guidelines.

### PLTP mRNA expression analysis

After systemic perfusion of mice with PBS, organs were excised, and total RNA was isolated (RNeasy mini kit, Qiagen) and used as a template for reverse transcription (iScript cDNA synthesis kit, Biorad). Quantitative PLTP RNA expression analysis was performed using a MyIQ 5.0 detection system (Biorad). A SYBR®Green PCR reagents kit (Eurogentec), and primers specific for human or murine PLTP were used (sequences available upon request). cDNA quantities were normalized to the amount of hypoxanthine–guanine phosphoribosyl transferase cDNA, using the ΔCt method [$2^{(-\Delta Ct)}$], and presented as arbitrary units (AUs). Averages were taken from at least three individual runs, each sample in triplicate.

### PLTP activity and mass

Plasma PLTP activity levels were determined by measuring the transfer of radiolabeled phospholipid from liposomes to exogenous HDL, as described previously (15), with a minor modification: liposomes were made using [$^{14}$C]-labeled instead of [$^{3}$H]-labeled phosphatidylcholine (Amersham). Plasma PLTP activity was expressed as AUs, where one AU equals 13.9 mmol/l/h, the level of PLTP activity that is found in human reference plasma. To determine the transfer of cholesterol, liposomes containing radiolabeled cholesterol (Amersham) were used. The transfer of α-tocopherol was determined using a micromethod with reverse-phase HPLC, with detection by fluorescence at 292/324 nm (21).

To determine hepatic phospholipid transfer activity levels, livers of PLTP tg, mutPLTP tg, and control mice were perfused in situ with ice-cold PBS, after which a section of approximately 0.2 g was homogenized in a buffer containing 50 mmol/l Tris-HCl (pH 7.4), 5 mmol/l EDTA, and 250 mmol/l sucrose. After centrifugation (16,000 *g*, 10 min, 4°C), supernatant was collected and phospholipid transfer activity was determined using the same protocol as for plasma PLTP. PLTP activity was calculated per milligram liver tissue, and expressed as AU. Hepatic phospholipid transfer activity of PLTP$^{-/-}$ mice (kindly donated by Dr. X-C. Jiang, Brooklyn, NY) was subtracted in order to express PLTP-specific activity.

Human PLTP mass in plasma was measured as described previously (22), using a sandwich-type ELISA using two monoclonal antibodies with specificity for human PLTP. The concentration of plasma PLTP was expressed as micrograms/milliliter.

### Separation of plasma lipoproteins

Plasma HDL fractions and non-HDL fractions were separated by ultracentrifugation of freshly isolated plasma samples at a density of 1.063 g/ml as described previously (16). Lipoprotein size distribution was determined by gel filtration chromatography. Plasma samples from 10 to 15 mice were pooled and passed through a 0.45 μm filter (Millipore), and 0.5 ml was applied onto a fast-protein liquid chromatography (FPLC) system containing a Superdex 200 prepgrade column and a Superose 6 prepgrade column connected in tandem. Columns were equilibrated with buffer containing 65 mmol/l sucrose, 225 mmol/l mannitol, 10 mmol/l Tris-HCl (pH 8.1), 5 mmol/l EDTA, and 0.02%

$NaN_3$ to obtain optimal PLTP recovery (23). After running (at 4°C, 0.1 ml/min), 0.8 ml fractions were collected and analyzed for total cholesterol (TC) levels and PLTP mass.

### Plasma liver lipid analyses

For the measurement of TC, a Free Cholesterol C kit from Wako was used, with cholesterol esterase from *Candida cylindracea* (Boehringer-Mannheim) to hydrolyze cholesteryl esters. Phospholipids were measured enzymatically with a PAP150 kit from BioMérieux. Triglycerides (TGs) were determined using a triglyceride kit from Wako.

### Determination of pre-β-HDL levels and formation

Pre-β-HDL levels were determined by crossed immunoelectrophoresis. Plasma samples were incubated for 5 h at 37°C in the presence of 1 mmol/l iodoacetate (IAA) to inhibit LCAT activity, or kept at 4°C in the absence of IAA. Five microliters of plasma was loaded per well. First-dimension agarose gel (1%) electrophoresis separated lipoproteins with pre-β- and α-mobility under nonreducing, nondenaturing conditions and low ionic strength. Second-dimension electrophoresis in a 1% agarose gel containing 7.5% polyclonal rabbit anti-mouse apoA-I antiserum quantified apoA-I in the samples. Gels were stained with Coomassie Brilliant Blue R250 and dried. Areas under pre-β- and α-HDL peaks were calculated for each condition. The relative pre-β-HDL area was expressed as a percentage of the total HDL area. Pre-β-HDL formation was determined as the difference between relative pre-β-HDL levels with and without incubation with IAA.

### VLDL secretion experiments

Following an overnight fasting period, mice were injected intravenously with 10% (w/v) Triton WR1339 (Tyloxapol, Sigma) dissolved in PBS, at a concentration of 500 mg/kg body weight. At different time points after injection, blood samples were taken from the orbital plexus, and plasma TG levels were determined as described above. The rate of TG secretion was calculated from the TG accumulation in time, and expressed as mmol/l/min.

### Quantification of atherosclerotic plaque size

After 13 weeks of the HFHC diet, mice were euthanized. Perfusion of hearts was performed in situ, with subsequent fixation using 4% PBS-buffered formalin. The hearts with aortic arch were excised and processed for cryosectioning. Serial 7 μm-thick sections of the valves in the aortic root were stained with Oil Red O and Mayer's hematoxilin. Atherosclerotic lesion area was measured in five sections at intervals of 90 μm using image processing by National Institutes of Health-based Scion Image and analyzing software (www.scioncorp.com) according to Paigen et al. (24). Mean lesion area per section was calculated for each mouse, and expressed as $\mu m^2$.

### Statistics

All values are expressed as mean ± SD. Differences were anayzed by two-sample Wilcoxon rank sum tests using Intercooled Stata 8.2/SE software (Stata Corporation, College Station, TX). Statistical significance was assumed when $P < 0.05$.

## RESULTS

### Expression of human PLTP in transgenic mice

To investigate the role of PLTP-mediated phospholipid transfer in vivo, the effects of overexpressing human PLTP with transfer activity and mutant human PLTP without transfer activity were compared in mice with an $LDLR^{+/-}$ background (PLTP tg and mutPLTP tg mice, respectively). First, we analyzed PLTP expression in PLTP tg, mutPLTP tg, and control ($LDLR^{+/-}$) mice.

RNA of different tissues (liver, lung, spleen, kidney, adrenals, and adipose tissue) was isolated and mRNA expression of murine and human PLTP was determined by real-time PCR. Human PLTP was expressed specifically in the liver of PLTP transgenic mice, whereas endogenous PLTP was expressed in all tissues tested (not shown). The overexpression of human PLTP in the liver did not affect endogenous PLTP mRNA levels, because these were not different between groups (**Fig. 1A**).

To check whether an increased PLTP expression in the liver was associated with changes in phospholipid transfer activity, we measured hepatic PLTP activity. PLTP activity in the livers of PLTP tg mice was approximately 2.5-fold higher than in mutPLTP tg and control mice. MutPLTP tg and control mice showed comparable activity levels (Fig. 1B).

Because PLTP is a protein secreted by the liver cells into the plasma, we quantified human PLTP protein levels as well as total PLTP activity levels in the mouse plasma. Because the assay to determine PLTP mass detects exclusively human PLTP, no signal was found in plasma from control mice. PLTP mass in mutPLTP tg mice was higher than in PLTP tg mice (Fig. 1C). Total plasma PLTP activity in PLTP tg mice was 2.9-fold higher than in control mice, whereas in mutPLTP tg mice, it did not exceed the endogenous mouse PLTP activity level (Fig. 1D). In addition to the reduced transfer activity of phospholipids, the transfer activity of cholesterol and α-tocopherol in plasma of mutPLTP tg mice was decreased compared with PLTP tg mice (cholesterol transfer in mutPLTP tg mice: 30% of transfer in PLTP tg mice; α-tocopherol transfer: 22% of transfer in PLTP tg mice; both $P < 0.05$), indicating that the mutation does not selectively affect phospholipid transfer.

### Effect of PLTP transfer activity on lipid metabolism

To determine whether the mutation influenced the ability of PLTP to associate with HDL, plasma lipoproteins were separated by FPLC, and TC as well as human PLTP mass were measured in the different fractions (**Fig. 2A**). Two major cholesterol peaks were distinguished in the plasma of both transgenic mouse groups, one peaking in fraction 8 and another in fraction 15, representing LDL and HDL particles, respectively. In both mouse models, human PLTP mass peaked in fractions 11–13, where a subfraction of large HDL particles, carrying PLTP, is present. These findings indicate that the in vivo association of PLTP protein with HDL particles was not affected by the mutation.

We studied the physiological role of PLTP transfer activity in lipid metabolism. TC, phospholipid, and TG were measured in total plasma samples and in plasma subfractions obtained after ultracentrifugation. Consistent with earlier findings, PLTP tg mice showed markedly decreased TC and HDL-cholesterol (HDL-C) levels compared with control mice (**Table 1**). In mutPLTP tg mice however, lipid values did not differ from those of control littermates. Gel

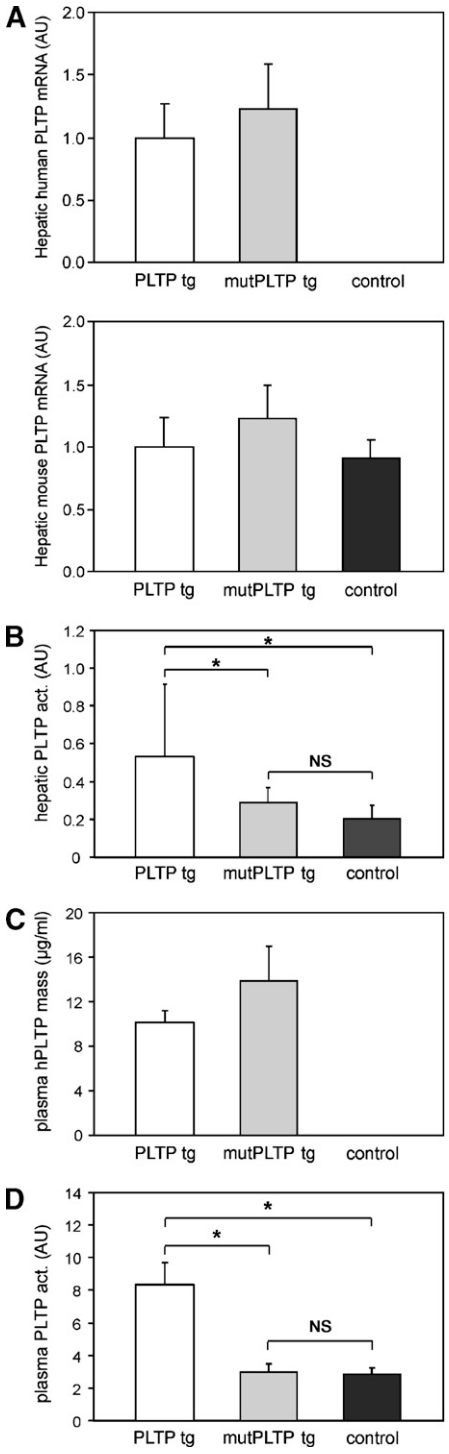


**Fig. 1.** Phospholipid transfer protein (PLTP) expression. A: Hepatic PLTP mRNA. The expression of human and mouse PLTP in livers of PLTP transgenic (PLTP tg), mutant PLTP transgenic (mutPLTP tg), and control mice was analyzed by real-time PCR. Values are expressed as arbitrary units (AUs) ± SD, n = 4 per group. B: Hepatic PLTP activity. PLTP activity in the liver is represented as the total phospholipid transfer activity in 1 mg liver tissue subtracted from the phospholipid transfer activity found in 1 mg liver tissue of PLTP-deficient mice. Values are expressed as AUs ± SD, n = 4–11 per group. *$P < 0.05$; NS, not significant. C: Plasma PLTP mass. Human PLTP protein was quantified using a sandwich-type ELISA. Values are expressed as µg/ml ± SD, n = 17–20 per group. D: Plasma PLTP activity. Values are expressed as AUs ± SD, n = 16–20 per group. *$P < 0.001$; NS, not significant.

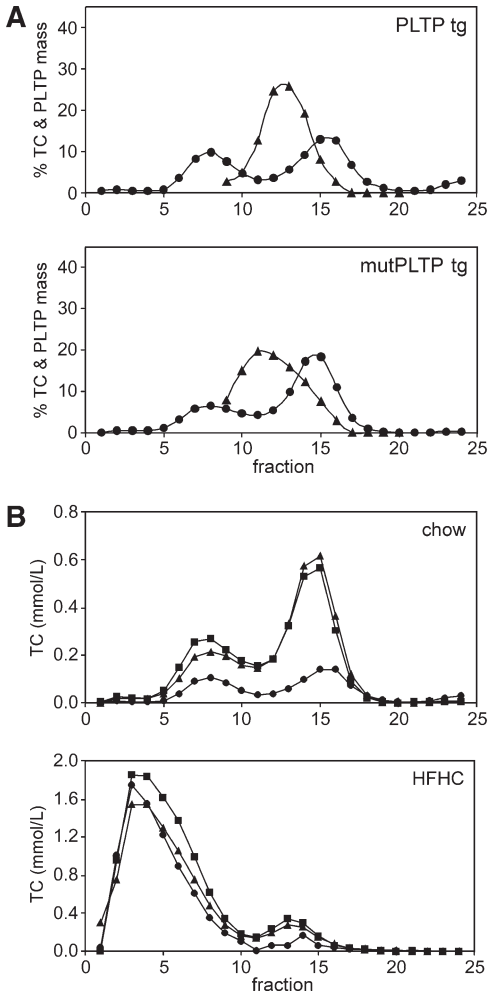


**Fig. 2.** Total cholesterol (TC) and PLTP distribution among plasma lipoprotein particles. Pooled plasma of PLTP tg, mutPLTP tg, and control mice (n = 10–15 per group) was subjected to fast-protein liquid chromatography (FPLC). A: TC (circles) and PLTP mass (triangles) in the FPLC fractions. Values are represented as fractional percentages of total amount of TC and PLTP mass measured in the column eluate. B: TC among lipoprotein particles in PLTP tg (circles), mutPLTP tg (triangles), and control mice (squares) on a chow or high-fat, high-cholesterol (HFHC) diet. Values are expressed as mmol/l.

filtration profiles confirmed these findings (Fig. 2B; upper graph). The same was found for phospholipid levels, whereas plasma TG levels were not different among the three groups (data not shown). To investigate whether the liver-specific overexpression of human PLTP in mice has consequences for liver lipid levels, cholesterol, phospholipid, and TG levels were determined in liver extracts. No significant differences between the mouse groups were observed (data not shown).

### Effect of PLTP transfer activity on pre-β-HDL formation and VLDL secretion

Previous studies have shown that PLTP enhances the formation of pre-β-HDL particles (15). To investigate the importance of PLTP transfer activity in this process, total plasma was incubated for 5 h with iodoacetic acid, an LCAT inhibitor, after which crossed immunoelectrophoresis ex-

TABLE 1. Plasma total cholesterol

| | HFHC diet | | |
|---|---|---|---|
| | 0 Weeks | 5 Weeks | 13 Weeks |
| | *mmol/l* | | |
| Plasma TC | | | |
| PLTP tg | 1.54 ± 0.24[a,b] | 9.90 ± 1.92 | 11.55 ± 3.74 |
| MutPLTP tg | 3.54 ± 0.36 | 11.61 ± 1.65 | 9.68 ± 2.38 |
| Control | 3.64 ± 0.49 | 11.10 ± 2.33 | 10.36 ± 2.86 |
| HDL-C | | | |
| PLTP tg | 0.65 ± 0.18[a,b] | 0.31 ± 0.05[a,b] | 0.30 ± 0.11[a,b] |
| MutPLTP tg | 2.10 ± 0.31 | 1.02 ± 0.29 | 1.18 ± 0.57 |
| Control | 2.18 ± 0.41 | 1.27 ± 0.31 | 1.38 ± 0.57 |
| non-HDL-C | | | |
| PLTP tg | 0.73 ± 0.24 | 9.01 ± 1.72 | 11.24 ± 3.81 |
| MutPLTP tg | 1.06 ± 0.07 | 9.66 ± 2.17 | 8.34 ± 2.18 |
| Control | 0.96 ± 0.12 | 9.24 ± 2.01 | 8.79 ± 2.87 |

HFHC, high-fat, high-cholesterol; TC, total cholesterol; PLTP, phospholipid transfer protein; PLTP tg, PLTP transgenic; mutPLTP tg, mutant PLTP transgenic; HDL-C, HDL cholesterol. Values are expressed as mean ± SD.

[a] $P < 0.001$ compared with control mice.

[b] $P < 0.001$ compared with mutPLTP tg mice.

periments were performed and mouse apoA-I was visualized (**Fig. 3**). In the absence of IAA, no pre-β-HDL could be detected in plasma (not shown). After incubation, 3.1% of total apoA-I was found in the pre-β-HDL subfraction in control mice (Fig. 3C) and 3.9% in mutPLTP tg mice (Fig. 3B), whereas in the PLTP tg mice, pre-β-HDL represented 28.1% of total plasma apoA-I (Fig. 3A), demonstrating the importance of active plasma PLTP for the production of pre-β-HDL.

To investigate the contribution of PLTP activity to the increased secretion of apoB-containing lipoproteins in mice with overexpression of PLTP, we performed VLDL-TG secretion experiments in vivo. As expected, TG secretion rates in PLTP tg mice were increased (1.4-fold) compared with those in control mice (**Fig. 4**). In contrast, mutPLTP tg mice without enhanced PLTP transfer activity did not stimulate VLDL secretion compared with control littermates. These findings show the role of active PLTP in hepatic VLDL secretion, whereas inactive PLTP has no effect.

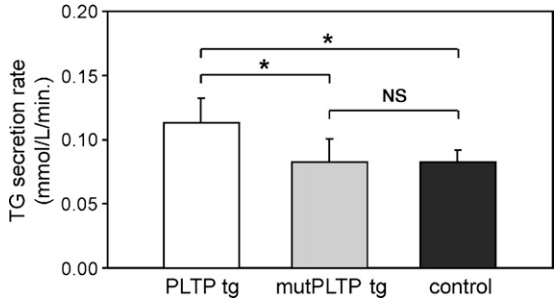


**Fig. 4.** Hepatic triglyceride (TG) secretion. Plasma TG levels were measured in plasma of PLTP tg (n = 6), mutPLTP tg (n = 23), and control mice (n = 12), at different time points after injection of mice with Triton WR1339. TG secretion rate was calculated using the slope of plasma TG secretion over time. Values are expressed as mmol/l/min ± SD. *$P < 0.005$; NS, not significant.

### Effect of PLTP transfer activity on plasma lipids and the development of atherosclerosis in mice on a HFHC diet

To study whether PLTP activity influences the development of atherosclerosis, mice were fed a HFHC, cholate-containing diet for 13 weeks. At regular time points after starting the diet, a blood sample was taken and lipid analyses were performed. After 13 weeks, mice were euthanized and the extent of atherosclerosis was quantified. The cholesterol-enriched diet increased TC and non-HDL-C, whereas it decreased HDL-C in all mice. PLTP tg mice showed a more profound decrease in HDL-C than did mutPLTP tg mice or control littermates (Table 1; Fig. 2B). PLTP activity was increased 1.5- to 2.5-fold in hyperlipidemic mice compared with mice on a chow diet ($P < 0.05$). The diet also induced PLTP mass levels, 1.5- and 1.7-fold in PLTP tg and mutPLTP tg mice, respectively ($P < 0.05$). After incubating plasma of hyperlipidemic mice for 5 h, pre-β-HDL levels (relative to total plasma HDL) increased by 13% in control mice (**Fig. 5F** versus 5C) and 15% in mutPLTP tg mice (Fig. 5E versus 5B). In PLTP tg mice, the formation of pre-β-HDL increased by 50%, and pre-β-HDL levels, which were already high before incubation, were close to 100% of total plasma HDL after incubation (Fig. 5D versus 5A). The

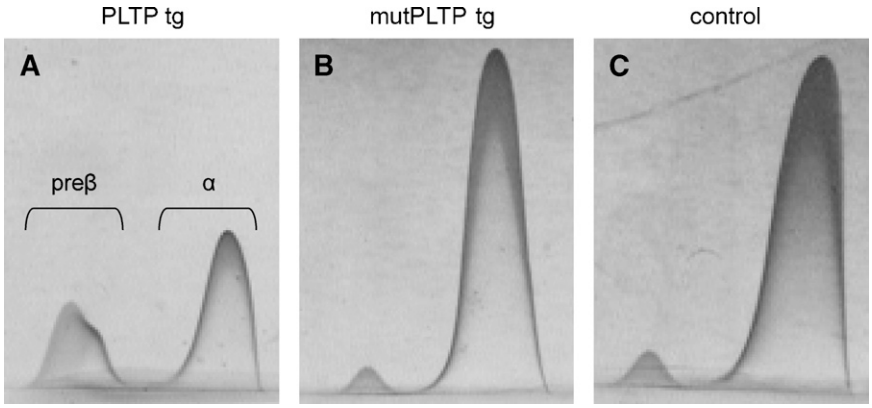


**Fig. 3.** Pre-β-HDL formation in mice on chow diet. Plasma of PLTP tg (A), mutPLTP tg (B), and control mice (C) was incubated with 1 mM iodoacetate (IAA) for 5 h at 37°C and subjected to crossed immunoelectrophoresis. Three to five mice per group were analyzed; one representative gel per group is shown. α- and pre-β-mobility of the HDL particles are indicated in A.

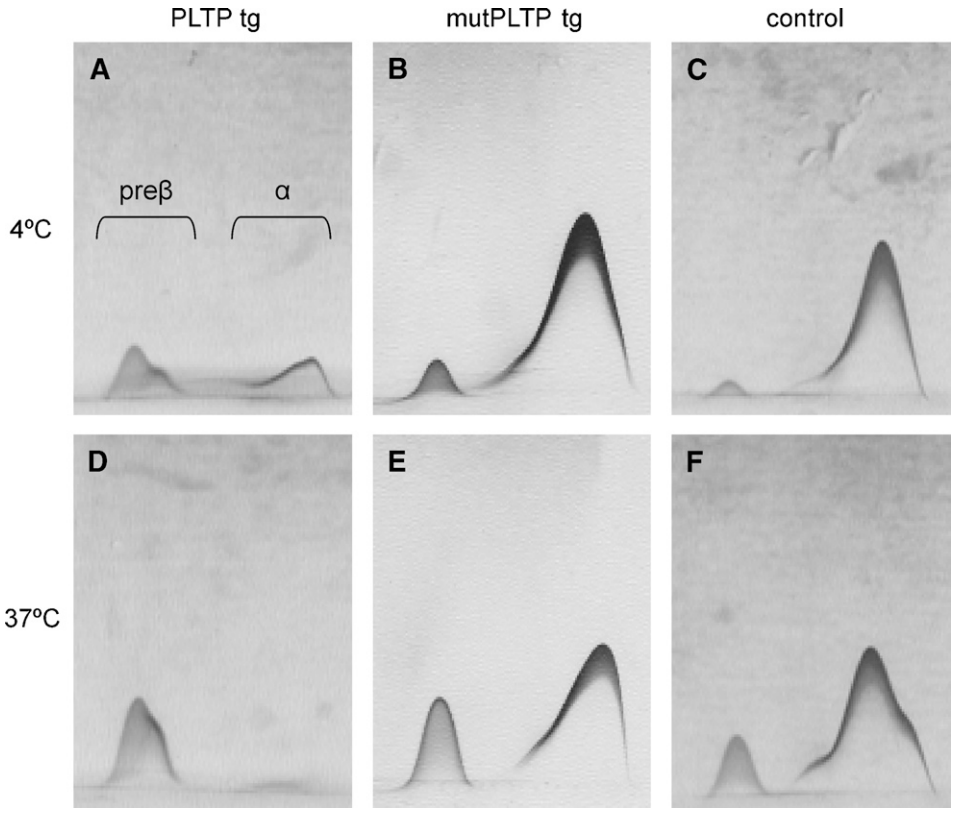


**Fig. 5.** Pre-β-HDL levels and formation in mice fed a HFHC diet. Plasma of PLTP tg (A, D), mutPLTP tg (B, E), and control mice (C, F) fed a HFHC diet for 13 weeks, was either kept at 4°C (upper panels), or incubated with 1 mM IAA for 5 h at 37°C (lower panels), after which it was subjected to crossed immunoelectrophoresis. Three to five mice per group were analyzed; one representative gel per group is shown. α- and pre-β-mobility of the HDL particles are indicated in A.

capacity of total plasma of hyperlipidemic mice to accept cholesterol from loaded macrophages in vitro, as investigated in cholesterol efflux experiments, was not different among the three groups (data not shown).

Finally, atherosclerotic lesion development was analyzed at the aortic valves. The overexpression of PLTP in PLTP tg mice increased the development of atherosclerotic lesions more than 2-fold compared with control mice (**Fig. 6**). In mice with overexpression of the mutant PLTP, atherosclerotic plaque sizes were not significantly different from those observed in control mice. These findings demonstrate that overexpression of PLTP with transfer activity accelerates the development of atherosclerosis, whereas mutant PLTP lacking transfer activity does not.

## DISCUSSION

In human plasma, a low-active form of PLTP has been described in addition to the high-active form (18). The role of this low-active PLTP, however, is not well understood. In a Japanese cohort study, a possible protective role for serum PLTP mass in coronary heart disease was indicated, independent of PLTP activity (25). Recently, it was published that distribution of PLTP between high-activity and low-activity forms may be disturbed in peripheral arterial disease (26). The few existing studies regarding the relationship between plasma PLTP activity and cardiovascular disease in humans show contradictory findings. Whereas Schlitt et al. (27) identified PLTP activity as an independent predictive value for coronary artery disease, others found variable relationships between PLTP activity and peripheral artery disease (26, 28).

In the present study, we aimed to elucidate the role of plasma PLTP transfer activity in *1*) the modulation of HDL levels, *2*) pre-β-HDL formation, *3*) hepatic VLDL secretion, and *4*) the development of atherosclerosis in PLTP transgenic mice. Hence, we generated a mouse model with liver-specific expression of a mutant human PLTP protein, associating with HDL particles in plasma and lacking phospholipid transfer activity, and cross-bred the animals in an $LDL^{+/-}$ background.

The liver-specific expression is the consequence of a PLTP transgene driven by the albumin promoter with enhancers. A similar construct was used in previous studies for the generation of PLTP tg mice (16). These PLTP tg mice developed similar levels of atherosclerosis compared with mice with equal plasma PLTP activity levels as a result of ubiquitous expression of human PLTP, showing that increased atherosclerosis development was caused by plasma PLTP activity. Therefore, the PLTP effects we evaluated in

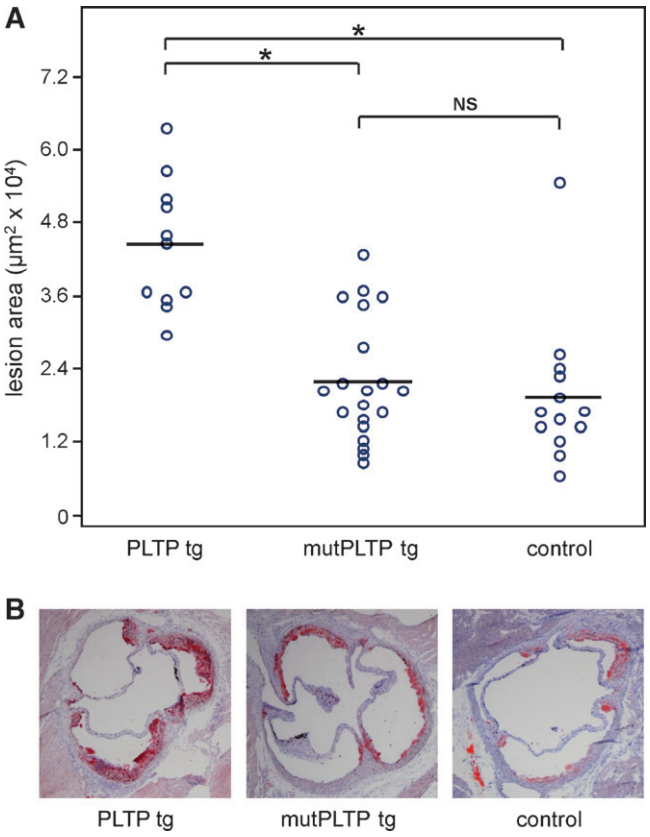


**Fig. 6.** Atherosclerotic lesion development Atherosclerotic lesion development was induced in PLTP tg (n = 11), mutPLTP tg (n = 20), and control (n = 13) mice by feeding a HFHC diet for 13 weeks. Cross sections of the aortic root were stained with Oil Red O to visualize the accumulation of lipids. A: Atherosclerotic lesion area of aortic valves, expressed as $\mu m^2 \times 10^4$ per section. Circles indicate individual lesion areas; horizontal lines indicate the mean lesion area per group. $*P < 0.001$; NS, not significant. B: Representative aortic root cross sections.

the present study represent systemic effects, caused by PLTP secreted by hepatocytes into the plasma.

We focused on the role of PLTP transfer activity in vivo by introducing the L196W mutation in the PLTP protein, based on a study from Huuskonen et al. (20). This mutation at the N-terminal lipid binding pocket of PLTP resulted in a decreased phospholipid transfer activity, without affecting the HDL binding characteristics of the protein. In plasma, PLTP is found associated with plasma HDL particles (29, 30). Therefore, when studying the role of PLTP activity in lipoprotein metabolism and atherogenesis, it is important to use a mouse model in which the mutation specifically affects the phospholipid transfer activity but not the PLTP-HDL association.

PLTP is a well-known HDL conversion factor. It remodels HDL with the generation of large α-HDL particles and small lipid-poor pre-β-HDL, by a mechanism that involves fusion of unstable HDL particles and displacement of lipid-poor apoA-I molecules in vitro (8, 31). HDL-associated PLTP has been shown to be an important regulator of plasma HDL levels in vivo. In PLTP transgenic mice, catabolism of HDL particles is enhanced (32), thereby decreasing plasma HDL levels. In the present study, we showed that this PLTP-dependent HDL-lowering effect requires increased PLTP transfer activity, and should therefore be distinguished from increased HDL catabolism leading to reduced HDL levels in PLTP-deficient mice. As revealed by crossed immunoelectrophoresis, the reduction in HDL levels in PLTP tg mice is mainly due to a decrease in α-migrating HDL particles. In addition to its effect on the catabolism of mature HDL particles, PLTP plays a role in the formation of pre-β-HDL particles, which also requires PLTP transfer activity. The PLTP effects on the distribution and levels of α- and pre-β-HDL were more pronounced when mice were fed a HFHC diet. This could be explained by the increased PLTP levels found in mice fed a high-fat diet. As a reaction to cholesterol loading, the production of pre-β-HDL might be stimulated, inasmuch as pre-β-HDL has been shown to be an excellent acceptor of cholesterol (33). We found that the capacity of total plasma of hyperlipidemic mice to accept cholesterol from loaded macrophages in vitro was not different among the three groups. The decreased α-HDL levels in plasma of PLTP tg mice may compensate for the increased pre-β-HDL levels regarding its role in cholesterol efflux in vitro. The role of systemic PLTP in cholesterol efflux in vivo is yet to be determined. The big decrease in α-HDL may override the potentially athero-protective effect of increased pre-β-HDL levels in PLTP tg mice in vivo.

When measuring hepatic phospholipid transfer activity, we found an increase in PLTP tg mice, possibly explaining the increase in VLDL secretion in these mice. In contrast, in mice expressing the mutated form of PLTP, neither hepatic phospholipid transfer activity nor VLDL secretion was altered compared with controls. Elevation of PLTP activity in the liver was not associated with differences in liver lipids, suggesting that hepatic PLTP is of minor importance for lipid homeostasis in the liver of PLTP transgenic mice. Moreover, in PLTP-deficient mice, hepatic phospholipid transfer activity is about 75% of the activity measured in wild-type mice (data not shown). This is not surprising, inasmuch as several other hepatic proteins able to transfer various types of phospholipids have been described (34).

Finally, we determined the importance of PLTP activity for the development of atherosclerosis in mice fed a cholesterol-enriched, cholate-containing diet. In the mutPLTP tg mice, the extent of atherosclerosis was comparable to that in the control littermates. In PLTP tg mice, however, increased atherosclerotic lesion sizes were found, consistent with earlier findings in mice overexpressing human PLTP (10, 16). The presence of cholate in the diet has been shown to regulate PLTP gene expression in vivo by the nuclear farnesoid X receptor (FXR), because the PLTP promoter contains an FXR response element (35). However, because we used an albumin promoter to generate our transgenic mouse models, it is not likely that plasma levels of human PLTP are affected by cholate feeding. Endogenous PLTP is possibly regulated by the cholate, but we expect that mouse PLTP levels were affected to the same extent in all mice. A limitation in our study is the presence of mouse PLTP, with activity, in all mice.

Whether PLTP mass, in the absence of any transfer activity, contributes to atherosclerosis development should be investigated in additional studies in mice by crossing the mice into a PLTP knockout background. However, we do not expect endogenous PLTP to influence our findings, inasmuch as plasma lipoprotein levels in mutPLTP tg*PLTP$^{-/-}$ mice are not different when compared with those in PLTP$^{-/-}$ mice (data not shown).

An important issue is how increased PLTP expression stimulates atherosclerosis. Elevation of PLTP activity (2.9-fold) results in HDL hypercatabolism and enhanced hepatic VLDL secretion. Both aspects may be important in the process of atherogenesis, and our present results suggest that PLTP transfer activity is required for these effects. In addition to the PLTP-mediated phopholipid transfer, the transfer of cholesterol and α-tocopherol was also inhibited. These transfer activities could account, at least in part, for the pro-atherogenic potential of PLTP in vivo. In addition, O'Brien et al. (36) suggested that PLTP may be pro-atherogenic by acting as a bridging protein between lipoproteins and biglycan, one of the major extracellular proteoglycans found in human atherosclerotic lesions. It is well known that retention of lipoproteins by extracellular matrix molecules is critical in the pathogenesis of atherosclerosis (36, 37). This bridging effect of PLTP has been shown to be independent of its phospholipid transfer activity in vitro (36). Furthermore, over the last 2 years, much attention has been paid to the functionality of PLTP in macrophages (38–40). However, results between different studies were contradictory, illustrating the complexity of the in vivo situation. Whereas Vikstedt et al. (40) demonstrated that PLTP deficiency in macrophages resulted in reduced plasma PLTP activity levels and a decreased atherosclerotic lesion development, two other research groups found an increased atherosclerosis development despite lower plasma PLTP activity levels, and suggested an atheroprotective role for macrophage-derived PLTP (38, 39). The relative contribution of systemic PLTP effects and local PLTP effects to atherosclerosis development remains unclear, and a balance between pro- and anti-atherogenic properties of PLTP might determine the impact of PLTP on atherosclerosis. In the present study, we showed the importance of plasma PLTP activity in the process of atherosclerosis development. Whether PLTP acts as a ligand binding to vascular proteoglycans, playing a role in the retention of HDL, and whether PLTP activity is required for local PLTP functions within the artery wall, needs further investigation. The use of an adapted version of our mutant PLTP mouse model, allowing expression of inactive PLTP in different tissues, might be very informative for future studies.

In conclusion, this study defines the contribution of PLTP transfer activity in plasma to atherogenesis in PLTP transgenic mice. Inhibitors of plasma PLTP activity could prevent atherosclerosis development in humans with elevated PLTP activity levels.

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