

Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion

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Abstract Two lipid transfer proteins are active in human plasma, cholesteryl ester transfer protein (CETP), and phospholipid transfer protein (PLTP). Mice by nature do not express CETP. Additional inactivation of the PLTP gene resulted in reduced secretion of VLDL and subsequently in decreased susceptibility to diet-induced atherosclerosis. The aim of this study is to assess possible effects of differences in PLTP expression on VLDL secretion in mice that are proficient in CETP and PLTP. We compared human CETP transgenic (huCETP^{tg}) mice with mice expressing both human lipid transfer proteins (huCETP^{tg}/huPLTP^{tg}). Plasma cholesterol in huCETP^{tg} mice was 1.5-fold higher compared with huCETP^{tg}/huPLTP^{tg} mice ($P < 0.001$). This difference was mostly due to a lower HDL level in the huCETP^{tg}/huPLTP^{tg} mice, which subsequently could lead to the somewhat decreased CETP activity and concentration that was found in huCETP^{tg}/huPLTP^{tg} mice ($P < 0.05$). PLTP activity was 2.8-fold increased in these animals ($P < 0.001$). The human PLTP concentration was 5 $\mu\text{g}/\text{ml}$. Moderate overexpression of PLTP resulted in a 1.5-fold higher VLDL secretion compared with huCETP^{tg} mice ($P < 0.05$). The composition of nascent VLDL was similar in both strains. These results indicate that elevated PLTP activity in huCETP^{tg} mice results in an increase in VLDL secretion. In addition, PLTP overexpression decreases plasma HDL cholesterol as well as CETP.—Lie, J., R. de Crom, T. van Gent, R. van Haperen, L. Scheek, I. Lankhuizen, and A. van Tol. Elevation of plasma phospholipid transfer protein in transgenic mice increases VLDL secretion. *J. Lipid Res.* 2002, 43: 1875–1880.

Supplementary key words PLTP • CETP • transgenic mice • VLDL

Phospholipid transfer protein (PLTP) is an important modulator of plasma HDL levels, size, and composition (1–5). HDL is considered to protect against atherosclerosis by transporting cellular cholesterol from cells in the arterial wall to the liver for further excretion via the bile, as well as by exerting anti-inflammatory and anti-oxidant effects (6–8).

The role of PLTP in atherosclerosis was recently evaluated in PLTP deficient mice (5). PLTP deficiency in hyperlipidemic mouse models resulted in decreased atherosclerosis. In vitro experiments with cultured hepatocytes from PLTP deficient mice revealed a defect in VLDL secretion. These effects on VLDL secretion provided an explanation for the decreased atherosclerosis found in PLTP deficient mice (5).

Earlier we reported anti-atherogenic properties in mice overexpressing human PLTP (huPLTP^{tg}). Despite lower HDL levels, plasma from these mice is more effective in preventing in vitro accumulation of cholesterol by macrophages and is able to generate more pre β -HDL (3, 9). Studies in mice with adenovirus-mediated overexpression of human PLTP showed similar effects on HDL subclass distribution (10, 11).

Thus, depending on the metabolic setting, PLTP may have anti- or pro-atherogenic properties that require further investigation. Presently, we aimed to evaluate whether VLDL secretion is affected by variations in PLTP activity. For this purpose, we crossbred transgenic mice for human CETP (huCETP^{tg}) with huPLTP^{tg} mice (9) and obtained huCETP^{tg}/huPLTP^{tg} mice. These mice provide a unique model to study the role of PLTP in VLDL metabolism in the presence of CETP, which by nature is the situation in humans. Plasma lipoproteins, plasma CETP, and PLTP activities, as well as human CETP and human PLTP mass were also measured to study the impact of PLTP in huCETP^{tg} mice.

METHODS

Breeding and treatment of transgenic mice

The huCETP^{tg} mice were kindly provided by Dr. A. R. Tall (Columbia University, New York) and are in C57BL6 background (9). HuPLTP^{tg} mice (3) were backcrossed to C57BL6 background for at least seven generations. Mice expressing both hu-

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man CETP and human PLTP (huCETP_{tg}/huPLTP_{tg}) were obtained by crossbreeding homozygous huCETP_{tg} with homozygous huPLTP_{tg} mice. Both transgenes have the natural flanking sequences, including the native promoters. Female mice were used in further experiments. Animals were housed under standard conditions with free access to water and regular chow diet.

After fasting overnight blood samples were collected from the orbital plexus by using VitrexTM sodium-heparinized micropipettes (80 IU) (Modulohm A/S, Copenhagen, Denmark) and immediately stored on ice. Blood was centrifuged at 2700 rpm for 15 min at 4°C. Plasma was either used directly or stored in small aliquots at -80°C before analysis. All experiments were approved by institutional and national guidelines (protocol nr.120.99.05).

In vivo hepatic VLDL secretion and composition

VLDL secretion experiments were performed according to Jong et al. (12). VLDL secretion was measured in overnight fasted mice which were injected intravenously with 15% (w/v) Triton WR1339 (Sigma, St Louis, MO) (500 mg/kg body weight) dissolved in 0.9% NaCl. After injection of Triton WR1339, blood samples were drawn at appropriate time points (up to 90 min) and triglyceride content was measured as described below. The triglyceride accumulation in plasma was linear during this time period. Hepatic triglyceride secretion rate was calculated from the slope of the line and expressed as $\mu\text{mol/h}$ per kg body weight.

From the blood samples obtained at $t = 90$ min after Triton WR1339 administration, plasma was taken and centrifuged at $d = 1.006$ g/ml in a Beckman 42.2 Ti rotor (34,200 rpm, 3 h, 12°C). The top fraction containing VLDL was isolated by tube slicing.

Quantification of plasma lipids and protein

Cholesterol was determined enzymatically with the Free Cholesterol C kit no. 274-47109 (WAKO, Neuss, Germany) after hydrolysis of cholesteryl esters with cholesterol esterase from *Candida cylindracea* (Boehringer, Mannheim, Germany). Triglycerides were measured with the Sigma GPO-Trinder kit no.337-B (Sigma, St Louis, MO) and free fatty acids were measured with the NEFA C kit no. 994-75409 (WAKO, Neuss, Germany). Phospholipids were measured with the PAP150 kit from Bio Merieux (Lyon, France). Protein was measured with a modification of the Lowry assay (13).

Separation of plasma lipoproteins by gel filtration

Plasma from transgenic mice was analyzed by gel filtration on two HR10/30 FPLC columns in tandem (Superdex 200 prepgrade, Superose 6 prepgrade, Pharmacia Biotech., Uppsala, Sweden). The columns were equilibrated with 2 mM $\text{NaH}_2\text{PO}_4/\text{Na}_2\text{HPO}_4$, pH 7.4 [containing 0.9% NaCl (w/v), 0.02% NaN_3 (w/v) and 5 mM EDTA]. Combined plasma samples from seven to ten mice were passed through 0.45- μm filters from Millipore S.A. (Molsheim, France), and 0.5 ml was subjected to gel filtration. The columns were run at 4°C with a flow rate of 0.1 ml/min. Fractions of 0.8 ml were collected. Recoveries were > 90% for all analyses.

Plasma activity assays

CETP and PLTP activity assays were performed according to Speijer et al. (14) as described before (9). The activities are expressed as percentage of human reference pool plasma. One hundred percent is equivalent to the following activities: CETP 215.6 nmol/ml/h; PLTP 13.9 $\mu\text{mol/ml/h}$.

Human CETP and human PLTP mass determinations by ELISA

The CETP ELISA assay was essentially performed as described by Mezdoor et al. (15). CETP mass was measured by a two-site an-

tibody immunoassay using a combination of two specific monoclonal antibodies for CETP: TP1 and TP2 (Ottawa Heart Institute, Canada), which are able to inhibit the transfer of cholesteryl esters as well as triglycerides and recognize a similar epitope localized in the carboxyterminal region of the CETP molecule (16). Both antibodies (10 $\mu\text{g/ml}$) were coated overnight by incubation in phosphate buffered saline (PBS, pH 7.4) at 4°C on microtiter plates (Immunoplate MaxiSorp; Nunc, Roskilde, Denmark). After washing the plates five times with washing buffer [PBS containing 6% (v/v) methanol and 0.2% (w/v) Thesit (Boehringer, Mannheim, Germany)], the plates were incubated for 1 h at 37°C with PBS containing 1% of bovine serum albumin (BSA; ICN, OH) to prevent non-specific binding. After incubation, the wells were washed five times and calibrated control plasma or mouse plasma samples, diluted in assaybuffer [washing buffer containing 1% (w/v) BSA], were added and incubated for 2 h at 37°C. After washing five times, antibody TP20 labeled with digoxigenine (Ottawa Heart Institute, Canada) was added and the plates were incubated for 2 h at 37°C followed by five times washing and incubation with a solution containing anti-digoxigenine Fab fragments for another 2 h at 37°C. Then, after washing five times, a solution with anti-digoxigenine coupled to peroxidase (Boehringer, Mannheim, Germany) was added followed by incubation for 45 min at 37°C. Tetramethylbenzidine (TMB, Merck, Darmstadt, Germany) was freshly prepared in DMSO (6 mg/ml). Five hundred microliters of the TMB/DMSO solution and 3 μl of H_2O_2 was added to 30 ml citrate buffer (35 mM, pH 5.5). One hundred microliters of this TMB solution was added to the wells after washing the plates five times. After 30 min, the reaction was stopped by the addition of 100 μl 2 N H_2SO_4 . Absorbance was read at 450 nm. The intra- and inter-assay coefficients of variation were 3.6% and 8.0% respectively. In the present study CETP activity correlated with human CETP mass ($r = 0.93$, $P < 0.001$).

Human PLTP mass was measured with a sandwich-type ELISA essentially as described previously (17) with minor modifications: the washing buffer contained 6% methanol (v/v) with 0.2% Thesit (w/v) (Boehringer, Mannheim, Germany) and 0.1% (w/v) Tween 20. The substrate solution used and the absorbance measurements were performed as described for the CETP mass determination. PLTP antibodies and standards were a generous gift from Dr. H. Hattori (BML incorporated, Saitama, Japan). In the present study PLTP activity correlated with human PLTP mass ($r = 0.84$, $P < 0.05$).

Statistical analysis

Data are expressed as mean \pm SD. Differences between huCETP_{tg} mice and huCETP_{tg}/huPLTP_{tg} mice were analyzed by two sample Wilcoxon rank-sum tests.

RESULTS

Plasma activities of CETP and PLTP

The activities of CETP and PLTP were measured in plasma samples from huCETP_{tg} mice and huCETP_{tg}/huPLTP_{tg} mice. CETP activity was 1.15-fold higher in huCETP_{tg} mice compared with huCETP_{tg}/huPLTP_{tg} mice ($P < 0.05$) (Table 1). PLTP activity in plasma of huCETP_{tg} mice was equal to activity levels found in wild-type mice (3). In huCETP_{tg}/huPLTP_{tg} mice PLTP activity was 2.8-fold higher ($P < 0.001$).

TABLE 1. Plasma levels of lipids, CETP, and PLTP activities as well as human CETP and human PLTP concentrations in transgenic mice

	n	huCETPtg	n	huCETPtg/huPLTPtg
Cholesterol (mM)	10	1.9 ^a ± 0.3	11	1.3 ± 0.1
Triglycerides (mM)	9	0.3 ± 0.4	5	0.2 ± 0.1
Free fatty acids (mM)	5	1.0 ± 0.1	5	1.0 ± 0.2
CETP activity (%)	12	126 ^b ± 13	11	110 ± 22
Human CETP (μg/ml)	6	4.4 ^b ± 1.9	7	2.9 ± 0.5
PLTP activity (%)	13	148 ^a ± 24	11	418 ± 54
Human PLTP (μg/ml)	4	ND	4	5.1 ± 0.7

CETP and PLTP activities are expressed as percentage of human reference pool plasma values (%). One hundred percent of the human reference pool plasma is equivalent to a CETP activity of 215.6 nmol/ml/h and to a PLTP activity of 13.9 μmol/ml/h. Differences between huCETPtg mice and huCETPtg/huPLTPtg mice were analyzed by two sample Wilcoxon rank-sum tests. Values are means ± SD. n, individual mouse plasma samples measured; ND, not detectable.

^a $P < 0.001$.

^b $P < 0.05$.

Lipoprotein analyses

Plasma samples from either huCETPtg or huCETPtg/huPLTPtg mice were analyzed by gel filtration chromatography to examine their lipoprotein profiles. Phospholipid contents as well as CETP and PLTP activities were determined in all fractions (Fig. 1). The HDL phospholipids in huCETPtg/huPLTPtg mice show a decrease due to PLTP overexpression (Fig. 1A). The phospholipids in fractions 20–25 represent lysophosphatidylcholine bound to albumin. A similar profile, apart from the peak in fractions 20–25, was obtained for cholesterol (not shown). CETP activity eluted in fractions corresponding to the size of HDL (Fig. 1B) and PLTP activity eluted in the fractions 9–15 corresponding with relatively large HDL particles (Fig. 1C), a situation also found in humans (14).

Determination of human CETP and human PLTP concentrations

Human CETP and human PLTP mass were analyzed in the plasma of huCETPtg and huCETPtg/huPLTPtg mice. CETP concentration was higher in huCETPtg mice compared with huCETPtg/huPLTPtg mice ($P < 0.05$). Human CETP mass co-eluted with CETP activity in the lipoprotein profiles of both huCETPtg and huCETPtg/huPLTPtg mice (Fig. 2A, B, respectively). PLTP concentration in huCETPtg/huPLTPtg mice was 5.1 ± 0.7 μg/ml. In huCETPtg/huPLTPtg mice human PLTP mass co-eluted with PLTP activity in the lipoprotein profile (Fig. 2C).

Cholesterol, triglyceride, and free fatty acid measurements

Before studying the influence of PLTP on VLDL metabolism, we measured cholesterol, triglyceride, and free fatty acid content in plasma of huCETPtg mice and huCETPtg/huPLTPtg mice. Plasma cholesterol in huCETPtg mice was 1.5-fold higher than in huCETPtg/huPLTPtg mice ($P < 0.001$) (Table 1). The triglyceride and free fatty acid levels were similar in both groups of mice (Table 1).

Effect of human PLTP on hepatic VLDL secretion

After intravenous injection of Triton WR1339, the rate of plasma triglyceride accumulation was measured by de-

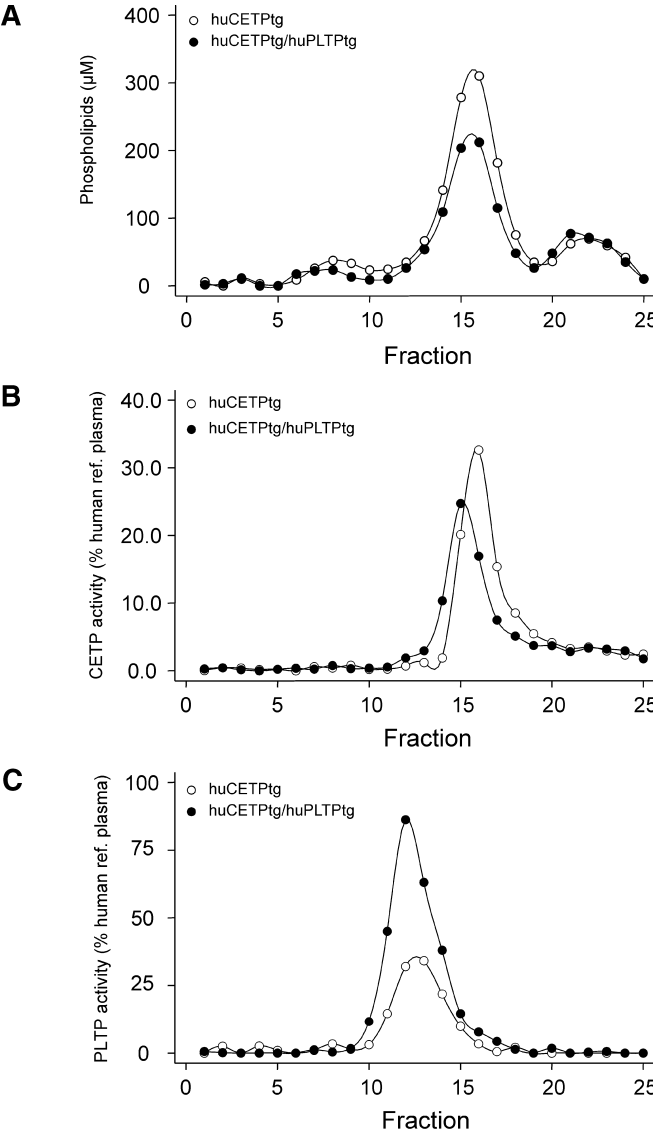


Fig. 1. Lipoprotein profiles of plasma from transgenic mice. Equal amounts of plasma from either huCETPtg mice ($n = 7$) or huCETPtg/huPLTPtg mice ($n = 10$) were pooled and subjected to gel filtration on Superose 6 and Superdex 200 columns connected in tandem as described in Methods. Fractions were analyzed for (A) phospholipids and (B) CETP activity: 100% human reference pool plasma is equivalent to CETP activity of 215.6 nmol/ml/h. C: PLTP activity: 100% human reference pool plasma is equivalent to PLTP activity of 13.9 μmol/ml/h. Fractions 1–5 contain VLDL; 6–11 contain LDL; 12–20 contain HDL and 21–25; contain lysophosphatidylcholine bound to albumin.

termining triglycerides in plasma at appropriate time points (Fig. 3A). The triglyceride accumulation rates calculated for each individual mouse were related to their body weights. There was no difference in body weight between the mice from the two groups (huCETPtg mice 19.6 ± 1.3 g; huCETPtg/huPLTPtg mice 19.2 ± 0.9 g). The secretion rate was calculated from the slope of the individual lines and is expressed as μmol/kg/h. The VLDL secretion rate was 1.5-fold higher in huCETPtg/huPLTPtg mice. HuCETPtg mice showed a rate of 140 ± 49 versus

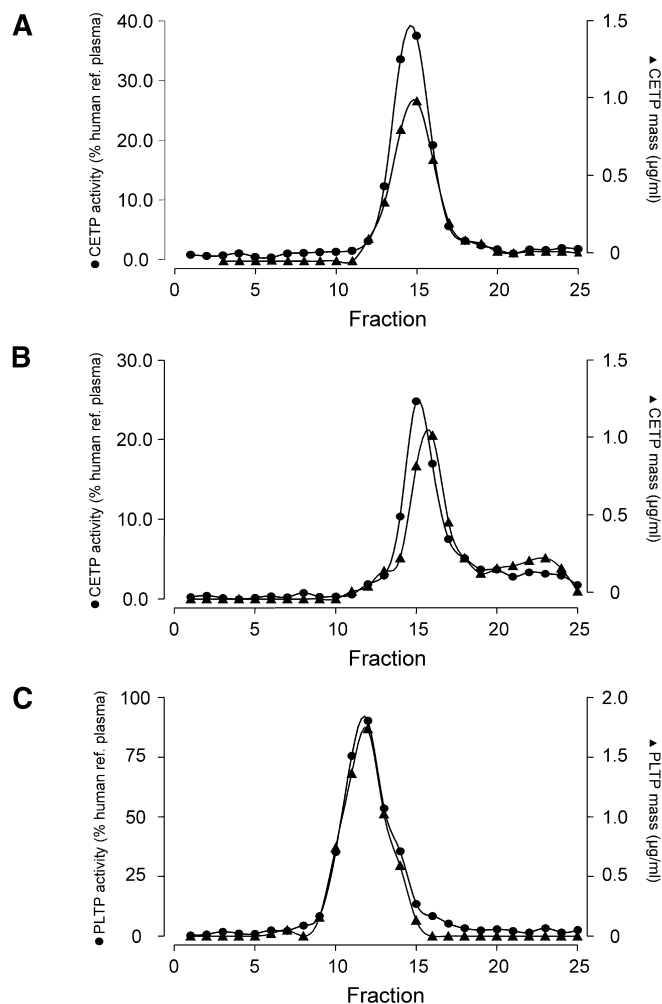


Fig. 2. Human CETP and PLTP concentrations with their corresponding activities measured in gelfiltration fractions of plasma from transgenic mice. A: Human CETP concentrations and CETP activities in huCETPtg mice ($n = 7$, $r = 0.99$, $P < 0.001$). B: Human CETP concentrations and CETP activities in huCETPtg/huPLTPtg mice ($n = 10$, $r = 0.92$, $P < 0.001$). C: Human PLTP concentrations and PLTP activities in huCETPtg/huPLTPtg mice ($n = 13$, $r = 0.97$, $P < 0.001$).

$208 \pm 41 \mu\text{mol/kg/h}$ in huCETPtg/huPLTPtg mice ($P < 0.05$).

The chemical composition of VLDL was analyzed at 90 min after Triton WR1339 injection. The composition of nascent VLDL (Fig. 3B) was similar in the two groups of mice. Others have verified that catabolism of VLDL is completely blocked by Triton WR1339 and therefore the composition of the accumulated VLDL is a direct measure of the composition of nascent VLDL (18).

DISCUSSION

The exact role of PLTP in lipoprotein metabolism is not clear. Earlier studies have shown that PLTP has both anti- and pro-atherogenic effects on lipoproteins (3, 5, 9). It was demonstrated that mouse models lacking PLTP are

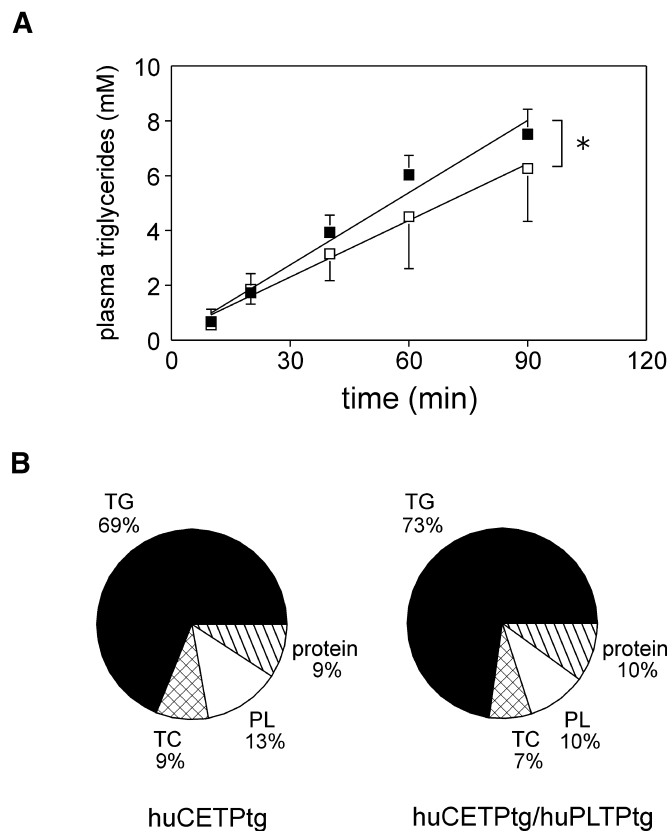


Fig. 3. In vivo hepatic VLDL secretion in transgenic mice. A: Triglyceride production rates in transgenic mice were measured after Triton WR1339 administration. HuCETPtg mice are represented by open squares ($n = 7$) and huCETPtg/huPLTPtg mice are represented by closed squares ($n = 6$). Differences between huCETPtg mice and huCETPtg/huPLTPtg mice were analyzed by two sample Wilcoxon rank-sum tests. The lines represent the mean triglyceride rates \pm SD of individual mice within huCETPtg and huCETPtg/huPLTPtg mice ($*P < 0.05$). B: Lipoprotein composition in nascent VLDL. VLDL was isolated from transgenic mouse plasma 90 min after Triton WR1339 administration by density ultracentrifugation at $d = 1.006 \text{ g/ml}$ (34,200 rpm, 3 h, 12°C). Lipids and protein were determined and expressed in percentage of total weight. Black segments represent the percentage of triglycerides, hatched segments represent the percentage of protein, white segments represent the percentage of phospholipids and cross-hatched segments represent the percentage of cholesterol in nascent VLDL.

less prone to diet-induced atherosclerosis. In two out of three models studied (5), this could be attributed to a reduced secretion of VLDL. PLTP deficiency or functional gene polymorphisms have not been found in humans. Moreover, unlike humans, mice do not have plasma CETP activity (19). Thus, total deficiency of both CETP and PLTP represent an extreme situation from which the effects on lipoprotein metabolism cannot be directly extrapolated to humans.

The purpose of the present study is to evaluate the influence of PLTP on VLDL metabolism in a mouse model with appreciable CETP activity. In this study, we demonstrate that elevated expression of PLTP increases hepatic VLDL secretion in mice in the presence of human CETP without affecting the composition of the secreted VLDL.

Despite the increase of VLDL secretion in the huCETPtg/huPLTPtg mice, we did not observe an increase in plasma VLDL levels. This may be due to an increased turnover of VLDL, or to increased receptor-mediated uptake by the liver. It is unlikely that the augmented VLDL secretion by PLTP is caused by an increased provision of free fatty acids from plasma to the liver, since no difference was found between the free fatty acid content in plasma of huCETPtg and huCETPtg/huPLTPtg mice (Table 1). Additional experiments showed that the elevation of VLDL secretion can be specifically attributed to the PLTP transgene. Using mice solely transgenic for human PLTP, we observed a 1.6-fold increase in VLDL secretion, if compared to wild type mice. As recently demonstrated (5), PLTP plays a possible intracellular role in the liver. Because PLTP activity has been found in the Golgi, PLTP could be involved in the process of adding lipids to nascent VLDL particles.

Plasma cholesterol content was 1.5-fold higher in huCETPtg mice compared with huCETPtg/huPLTPtg mice (Table 1). Thus, overexpression of human PLTP in huCETPtg mice results in a decrease in total plasma cholesterol, mostly due to HDL lowering, as HDL is the major component of the plasma lipoproteins in mice. Raised PLTP activity in other transgenic mouse models also give rise to low HDL cholesterol levels (3, 10, 11). The decrease in plasma HDL is explained by an enhanced uptake of HDL cholesteryl esters by the liver (10). Interestingly, PLTP deficient mice also show markedly reduced HDL, probably due to HDL hypercatabolism (20, 21). Kawano et al. demonstrated that CETP overexpression could not compensate PLTP deficiency and caused an additional lowering of HDL (22).

Plasma CETP activity measured in huCETPtg mice is higher than in huCETPtg/huPLTPtg mice (Table 1). The lower CETP activity found in huCETPtg/huPLTPtg mice may result from the lower plasma HDL concentrations caused by PLTP overexpression (9). Because CETP is carried on HDL (14), lower plasma HDL concentrations could eventually give rise to lower CETP levels. The CETP concentrations in both groups of mice (Table 1) are within the range of human values, indicating that the mouse models used resemble the human situation both in terms of CETP activity and mass (23).

PLTP activity levels in plasma of huCETPtg were equal to the activities found in wild-type mice (3). As expected, PLTP activity was higher in huCETPtg/huPLTPtg mice than in the huCETPtg mice (Table 1). The human PLTP mass measured in huCETPtg/huPLTPtg mice is within the range of reported plasma PLTP concentrations in humans (17, 24, 25). In contrast to what has been reported in humans (26, 27), in our mice PLTP activity correlates with human PLTP mass. In human plasma, such a correlation is not found due to the presence of inactive PLTP mass. In our studies, we found no evidence of inactive forms of PLTP in mice, since PLTP activity and PLTP mass elute in the same fractions in the lipoprotein profile (Fig. 2C). At present, the physiological importance, if any, of inactive PLTP is unknown. Studies in humans showed that type II diabetic patients have higher PLTP mass and activ-

ity compared with nondiabetic subjects (24, 28). Increased PLTP activity has also been reported in type I diabetic patients (29) and in the obese (30, 31). Both diabetes and obesity have been associated with an increased risk of coronary artery disease, indicating a possible pro-atherogenic potential for PLTP.

By comparing huCETPtg mice with huCETPtg/huPLTPtg mice we observed that elevated PLTP leads to increased hepatic VLDL secretion. This is in line with experiments by Jiang et al. performed with cultured hepatocytes isolated from PLTP deficient mice, which showed a defect in VLDL secretion (5), and strengthens their suggestion that PLTP has an intracellular function in liver cells.

In the present study, we showed for the first time that elevation of plasma PLTP in transgenic mice increases VLDL secretion. Concomitantly with increased VLDL secretion, elevated PLTP resulted in lower levels of plasma HDL, both effects resulting in a more atherogenic lipoprotein profile. The huCETPtg/huPLTPtg mice used in this study provide a unique model because it resembles the human condition in terms of CETP activity and mass. In future studies we will test the impact of PLTP overexpression on diet induced atherosclerosis in huCETPtg mice.

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