

Adenovirus mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice¹

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Abstract To study the function of plasma phospholipid transfer protein (PLTP) *in vivo*, a liver directed adenoviral gene transfer system was used to overexpress human PLTP in mice. For the experiments, two strains of mice, wild type (C57/Bl) and mice transgenic for the human apoA-I gene (HuApoA-ITg), were utilized. Five days after injection of the recombinant PLTP adenovirus, wild type mice showed a 4-fold increase in serum PLTP activity in ($12.2 \pm 1.3 \mu\text{mol/ml/per h}$ to $48.1 \pm 8.6 \mu\text{mol/ml per h}$ (+394%), $P < 0.001$). The PLTP overexpression induced significant reduction of serum cholesterol (2.46 ± 0.08 to 0.69 ± 0.42 mmol/l (-72%), $P < 0.001$), phospholipids (3.10 ± 0.06 to 0.90 ± 0.24 mmol/l (-71%), $P < 0.01$), and triglycerides (0.2 ± 0.07 to 0.08 ± 0.03 mmol/l (-69%), ($P < 0.001$). ApoA-I was hardly detectable in the serum. These lipid changes were due to a dramatic reduction of high density lipoprotein (HDL). The HuApoA-ITg mice displayed higher basal HDL level and PLTP activity. Adenovirus mediated PLTP overexpression in these mice resulted in a similar decrease of the lipid levels as that seen in the C57/Bl mice. However, the lipoprotein profile revealed a redistribution of HDL, with the appearance of larger buoyant HDL species. The results demonstrate that plasma phospholipid transfer protein *in vivo* causes high density lipoprotein (HDL) conversion and thereby plays a central role in HDL metabolism.—Ehnholm, S., K. Willems van Dijk, B. van 't Hof, A. van der Zee, V. M. Olkkonen, M. Jauhiainen, M. Hofker, L. Havekes, and C. Ehnholm. Adenovirus-mediated overexpression of human phospholipid transfer protein alters plasma HDL levels in mice. *J. Lipid Res.* 1998. 39: 1248–1253.

Supplementary key words recombinant adenovirus • phospholipid transfer protein • HDL metabolism • HDL conversion • apolipoprotein A-I • lipid metabolism

Although epidemiological studies have shown an inverse correlation between high density lipoprotein (HDL) cholesterol levels and cardiovascular disease (for review, see 1, 2), the cardioprotective mechanism of HDL is not

well understood. An increasing amount of evidence suggests that the antiatherogenic potential of HDL involves HDL-mediated removal of cholesterol from cell membranes of the arterial wall and subsequent transport of the lipid to the liver for excretion, a process named reverse cholesterol transport (3).

The HDL fraction consists of a heterogeneous population of particles differing in size, density, electrophoretic mobility, and composition (4). These HDL subpopulations display metabolic and functional differences. It has been shown that small apoA-I-rich HDL particles with pre β -mobility act as the primary acceptors of free cholesterol from peripheral cells *in vitro* (5, 6). Accordingly, the distribution of HDL subclasses *in vivo* is of great importance in the evaluation of HDL anti-atherogenicity. HDL particles are constantly modified in the circulation by enzymes, such as lipoprotein lipase (LPL), hepatic lipase (HL), and lecithin-cholesterol acyltransferase (LCAT), as well as the plasma lipid transfer proteins, cholesteryl ester transfer protein (CETP) and phospholipid transfer protein (PLTP).

PLTP is a 476 amino acid hydrophobic glycoprotein (7). The mouse and human proteins share 83% identical amino acids, as deduced from the cDNA sequence (5, 8). PLTP belongs to the protein family consisting of CETP, lipopolysaccharide binding protein, and bactericidal per-

Abbreviations: HDL, high density lipoprotein; LDL, low density lipoprotein; IDL, intermediate density lipoprotein; VLDL, very low density lipoprotein; PLTP, phospholipid transfer protein; apo, apolipoprotein; ad-PLTP, human PLTP recombinant adenovirus; adLacZ, β -galactosidase recombinant adenovirus; HuApoA-ITg, mouse transgenic for human apoA-I; LPL, lipoprotein lipase; HL, hepatic lipase; LCAT, lecithin:cholesterol acyltransferase; CETP, cholesterol ester transfer protein; pfu, plaque forming unit

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meability-increasing protein (7, 9). In vitro studies have shown that PLTP is able to transfer different phospholipids (10–12), α -tocopherol (13), lipopolysaccharide (14) and free cholesterol (15), between lipoprotein particles or between lipoproteins and reconstituted vesicles. In addition, PLTP is capable of modifying the HDL particle size distribution, a process termed HDL conversion (16–18). Although these in vitro observations suggest that PLTP may play an important role in HDL metabolism in vivo, very little is known about its actual physiological function. Two recent reports on transgenic mice overexpressing human PLTP (19, 20) have addressed this question. Both studies demonstrated that overexpression of human PLTP in transgenic mice had an influence on HDL metabolism. However, the expression levels reached were low and the results somewhat contradictory.

In order to elucidate the role of PLTP in lipoprotein metabolism in vivo, we overexpressed human PLTP in two strains of mice using an efficient adenoviral vector system. The effects of PLTP overexpression on the lipid and lipoprotein parameters of the infected mice were analyzed. Our study demonstrates that PLTP overexpression in wild-type and in human apoA-I transgenic mice significantly reduces serum cholesterol levels. In both mouse strains the cholesterol change was mainly due to a reduction in plasma HDL. Further, HDL remodelling reminiscent of the PLTP mediated HDL conversion in vitro was observed.

MATERIAL AND METHODS

Recombinant adenovirus

The adenoviral adaptor plasmid pCMV-PLTP was generated from the plasmids pCMV10 (supplies the human adenovirus type 5 (Ad5) 5'-inverted terminal repeat, the Ad5 origin of replication, the Ad5 encapsidation signal, cytomegalovirus (CMV) immediate early promoter, SV40 19s exon, SV40 truncated intron and a splice acceptor site) and pMLP-TK (supplies a polyadenylation site and Ad5 sequences from map unit 9.2–16.9 to serve as homologous recombination fragment), kindly provided by Introgene BV (Leiden, the Netherlands). The human PLTP cDNA (11) was inserted immediately downstream of the splice acceptor site, thus generating the pCMV-PLTP vector. The recombinant adenovirus was generated by cotransfection of pCMV-PLTP with pJM17 (21) into 911 cells (22) using a calcium phosphate transfection kit (Promega). Ten to 12 days after transfection, recombinant adenoviral plaques were picked and screened for the presence of the PLTP cDNA by polymerase chain reaction (PCR). Positive plaques were amplified on 24-well dishes and the supernatant of lysed wells was assayed for PLTP activity. A 10-fold increase in PLTP activity in the medium was measured as compared to the the medium of non-infected cells.

The recombinant adenovirus expressing the β -galactosidase gene under control of the CMV promoter (adLacZ) was kindly provided by Dr. J. Herz (23).

The recombinant adenoviruses were propagated and titrated as previously described (24). For in vivo adenovirus transfection, the virus was purified twice by CsCl gradient centrifugation, followed by extensive dialysis against TD (25 mM Tris-HCl, 137 mM NaCl, 5 mM KCl, 0.73 mM Na_2HPO_4 , 0.9 mM CaCl_2 , 0.5 mM MgCl_2 , pH 7.45), at 4°C. After dialysis, mouse serum albumin was

added to 0.2% and glycerol to 10%, and virus stocks were stored in aliquots at -80°C . The titers of the viral stocks were $1\text{--}6 \times 10^{10}$ plaque forming units (pfu) per ml.

Animals

The C57/Bl6 mice (C57/Bl) were purchased from Broekman Instituut B.V. (Someren, The Netherlands). The human apoA-I transgenic (HuApoA-ITg) mice were kindly provided by Dr. E. Rubin (25). Female mice 6–15 weeks of age were used for the experiments. Mice were kept with free access to water and a standard mouse-rat (chow) diet. Blood samples were drawn after a 4-h fast.

Two to 3 days before adenovirus injection, baseline lipid values were measured. On day zero, mice were injected into the tail vein with 6×10^8 pfu of recombinant adenovirus diluted with PBS to a total volume of 200 μl . Blood samples were drawn on days 3 and 5 after injection. On day 5 the mice were killed. Serum was isolated from the blood after 30 min incubation at 37°C , followed by 10 min incubation at 4°C and two rounds of centrifugation at 4000 rpm in an Eppendorf centrifuge. The serum was kept at 4°C until analysis.

PLTP activity and protein analysis

PLTP activity was measured using the radiometric assay described by Damen, Regts, and Scherphof (26) with minor modifications (16, 18).

For detection of the PLTP protein by immunoblotting, 4 μl of mouse serum was applied to a 12.5% SDS-PAGE gel under reducing conditions. After electrophoresis the proteins were transferred to a nitrocellulose membrane (Schleicher & Schuell no. 401196) and treated with a polyclonal rabbit antibody against human PLTP. The bound antibodies were detected using peroxidase labeled goat anti-rabbit IgG (Bio-Rad no. 170-6515). The PLTP antibody was produced in rabbits using a synthetic peptide antigen comprising amino acids 453–476 of the protein (synthesized by Chiron Mimotopes Peptide Systems, Victoria, Australia).

Assay of LCAT activity

Serum LCAT activity was determined as previously described (27).

Plasma lipid and lipoprotein analysis

Total serum cholesterol (Boehringer Mannheim, cat. no. 236691), triglycerides (Sigma Triglyceride (GPO-Trinder) cat. no. 337-B), phospholipids (Waco, Phospholipids B code 990-54009), and free cholesterol (Boehringer Mannheim cat. no. 310328) were measured with enzymatic methods. Cholesterol ester concentration was calculated by subtracting the amount of free cholesterol from the total plasma cholesterol.

Serum lipoproteins were isolated by density gradient ultracentrifugation according to Redgrave, Roberts, and West (28). Briefly, 200 μl of pooled mouse serum (for wild-type mice, $n = 5$; for HuApoA-ITg mice, $n = 4$) was subjected to density gradient ultracentrifugation (SW41 rotor, 4°C , 40,000 rpm, 19 h), after which the gradient was fractionated into 0.5-ml fractions. The density of the fractions was measured using a DMA 602M densitometer (Paar, Germany). Fractions with density < 1.006 , 1.006–1.019, 1.019–1.063, and 1.063–1.21 g/ml correspond to VLDL, IDL, LDL, and HDL, respectively. The cholesterol, triglycerides, and phospholipids in the fractions were determined as above.

To analyze the apolipoprotein composition, the fractions were dialyzed against phosphate-buffered saline (PBS) containing 1 mM EDTA (pH 7.4), and 30 μl of the dialyzed fractions was analyzed on 4–20% gradient SDS-PAGE gels under reducing conditions. Parallel gels were run for each sample. One was stained with Coomassie Brilliant blue and the other one was immunoblotted using polyclonal antibodies against human or mouse anti-

apoA-I. Peroxidase-labeled goat anti-rabbit IgG (Nordic Immunity, Tilburg, The Netherlands) was used for detection.

The size of the HDL particles was determined by native gradient gel electrophoresis (29), using the modifications of Blomqvist et al. (30).

Liver function was followed by determining serum alanine aminotransferase (ALAT) activity.

Statistical analysis

Serum lipid levels and circulating enzyme activities were compared using ANOVA and student's *t*-test. To determine the significance of differences between groups, Student's *t*-test was used.

RESULTS

PLTP activity in the serum of mice injected with recombinant adenovirus

Two strains of mice were used in the overexpression studies. In addition to wild type (C57/Bl) mice, human apoA-I transgenic mice were used (HuApoA-ITg), as it has previously been reported that the activity of CETP, LCAT, and also PLTP is enhanced when they are expressed in mice transgenic for the human apoA-I gene (19, 31, 32).

Before injection of recombinant adenovirus, the endogenous serum PLTP activity in C57/Bl mice was 9.4 ± 1.2 $\mu\text{mol/ml per h}$ and in the HuApoA-ITg mice somewhat higher, 22.0 ± 2.2 $\mu\text{mol/ml per h}$. Injection of the adPLTP virus (6×10^8 pfu) in the tail vein resulted in a significant elevation of serum PLTP activity on day 5 (Table 1). In the C57/Bl mice up to 4-fold and in the HuApoA-ITg mice a 2.5-fold increase was observed. In control mice injected with adLacZ, PLTP activity was not significantly changed. Injection of adPLTP resulted in the presence

TABLE 1. Serum lipids, human apoA-I, and PLTP activity levels in mice 5 days post injection of recombinant PLTP adenovirus (adPLTP) or recombinant LacZ adenovirus (adLacZ)

Variable	AdLacZ	AdPLTP	Δ -(%)
C57/Bl, n = 5			
TC	2.46 ± 0.04	0.69 ± 0.19	72 ^a
CE	1.92 ± 0.03	0.39 ± 0.19	70 ^b
FC	0.54 ± 0.03	0.30 ± 0.11	44
FC/CE	0.31 ± 0.03	1.37 ± 0.63	77
PL	3.10 ± 0.06	0.90 ± 0.24	71 ^b
TG	0.25 ± 0.03	0.08 ± 0.01	68 ^a
PLTP activity	12.0 ± 0.6	48.1 ± 3.9	75 ^a
Hu apoA-I	0	0	0
HuApoA-ITg, n = 4			
TC	4.27 ± 0.25	2.38 ± 1.29	44 ^c
CE	2.95 ± 0.20	1.79 ± 0.53	39
FC	1.31 ± 0.23	0.59 ± 0.12	55 ^c
FC/CE	0.47 ± 0.12	0.48 ± 0.19	4
PL	2.30 ± 0.20	0.90 ± 0.36	61 ^b
TC	0.34 ± 0.07	0.11 ± 0.06	68
PLTL activity	22.2 ± 1.5	58.5 ± 1.7	62 ^a
Hu apoA-I	3.48 ± 0.15	1.10 ± 0.10	68 ^a

TC, total cholesterol; CE, cholesteryl esters; FC, free cholesterol; PL, phospholipids; TG, triglycerides. All lipid values are given as mmol/l, PLTP activity as $\mu\text{mol/ml per h}$, and human apoA-I as mg/ml. All values are given as mean \pm SEM.

^a $P < 0.001$.

^b $P < 0.001$ -0.01.

^c $P = 0.01$ -0.05.

of human PLTP protein in the serum of the mice, as determined by immunoblot analysis with a rabbit anti-PLTP antibody. The adenoviral infection did not affect liver function in the mice as judged from the ALAT levels (data not shown).

Effect of human PLTP overexpression on mouse serum lipids and lipoproteins

Determination of serum lipid values was performed 5 days after injection of recombinant adenoviruses. The C57/Bl mice that received adPLTP displayed a 72%, 68%, and 71% decrease in the total serum cholesterol, triglycerides, and phospholipids, respectively, as compared to the mice that were injected with adLacZ (Table 1). The ratio of free cholesteryl to cholesteryl esters in the adPLTP-injected mice was clearly elevated compared to the adLacZ-injected group (1.4 vs. 0.3). Serum LCAT activity in the adPLTP-injected mice was significantly reduced (73% decrease on day 5 post-injection) compared to the adLacZ-injected mice (Fig. 1).

Overexpression of human PLTP in the HuApoA-ITg mice resulted in a decrease of serum lipids comparable to that observed in the C57/Bl mice. The decrease in total cholesterol, triglycerides and phospholipids was 44%, 50%, and 61%, respectively. The concentration of human apoA-I was also significantly decreased in these mice (68%). The injection with the control virus, adLacZ, did not induce significant lipid changes in either of the mouse strains studied.

Effect of PLTP overexpression on the mouse lipoprotein profiles

Analysis of the lipoprotein distribution by density gradient ultracentrifugation revealed a marked decrease of the HDL fraction in both mouse strains upon overexpression of PLTP (Fig. 2). The C57/Bl mice injected with adPLTP displayed a marked reduction of cholesterol, triglycerides, and phospholipids throughout the fractions. However, the relative proportion of phospholipids was elevated in the dense

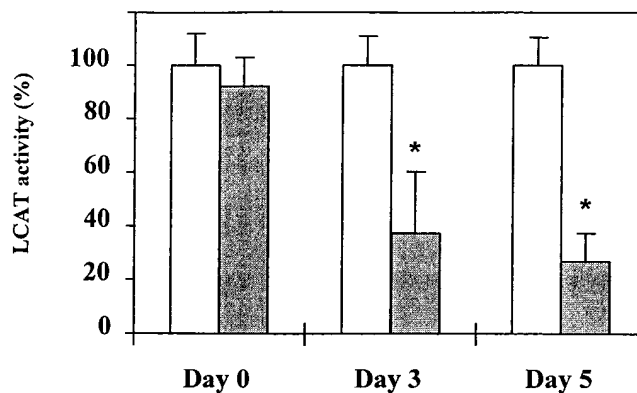


Fig. 1. The effect of PLTP overexpression on the serum LCAT activity in C57/Bl mice. The results (mean \pm SEM, n = 4) are expressed relative to the activity present in adLacZ mice. LCAT activity was measured on days 0, 3 and 5; \square , adLacZ; \blacksquare , adPLTP. *Significantly different from the adLacZ mice (Student's *t*-test, $P < 0.01$)

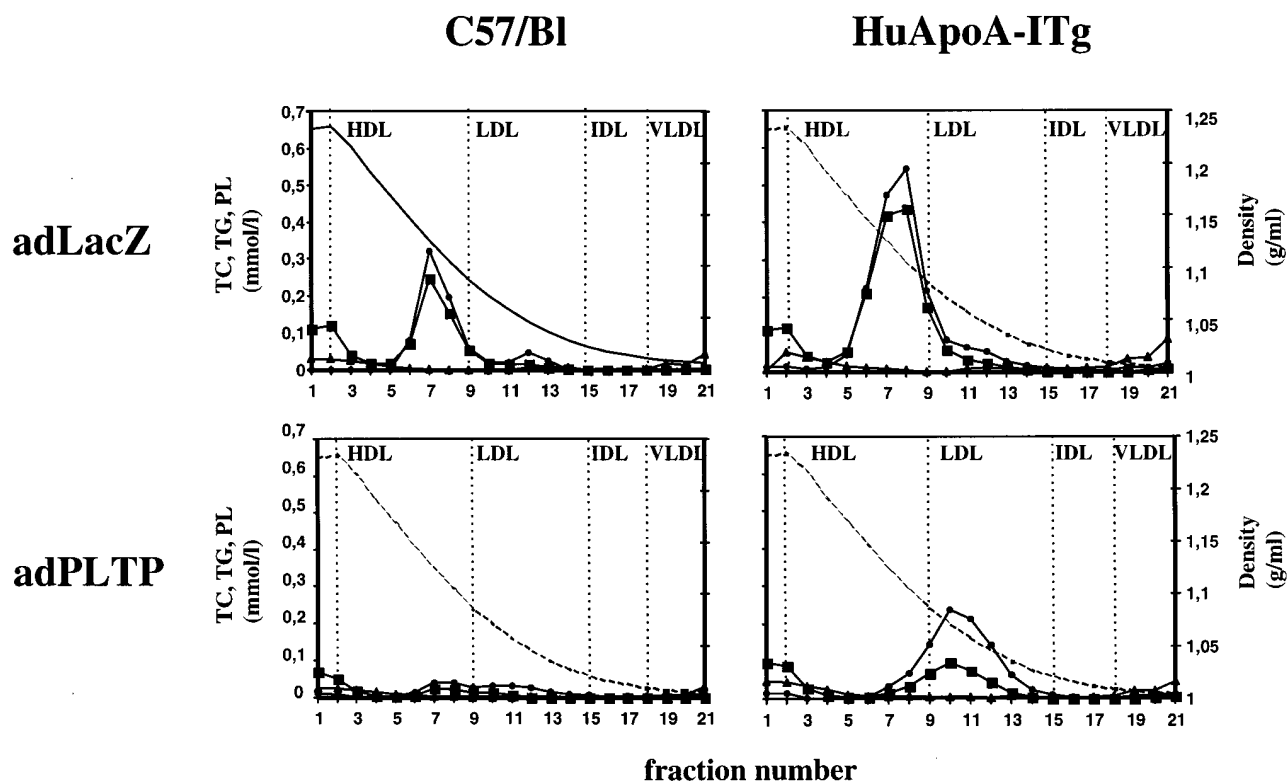


Fig. 2. Cholesterol, phospholipid and triglyceride distribution in lipoprotein fractions separated by density gradient ultracentrifugation. Serum samples were collected 5 days after recombinant adenovirus injection. Cholesterol (●-●), phospholipids (■-■), and triglycerides (▲-▲). The density is displayed with the dashed line (----). Ultracentrifugation gradient fractions are indicated by numbers at the bottom.

bottom fractions ($d > 1.21$ g/ml) in the adPLTP-injected mice (Fig. 2). The amount of apoA-I in the HDL density range was decreased in mice injected with adPLTP (Fig. 3).

The HuApoA-ITg mice also displayed a marked decrease in HDL cholesterol, triglycerides and phospholipids upon adPLTP injection (Fig 2). However, the change was not as dramatic as in the C57/Bl mice, and the HDL

peak was clearly detectable. The HDL displayed a shift towards lighter density, the peak overlapping with the LDL density range (Fig. 2). Phospholipids were distributed between the buoyant lipoprotein particles (59.9% of total) and the bottom fractions $d > 1.21$ g/ml (36.1% of total). Protein analysis by SDS-PAGE with Coomassie staining and Western analysis demonstrated that the main apopro-

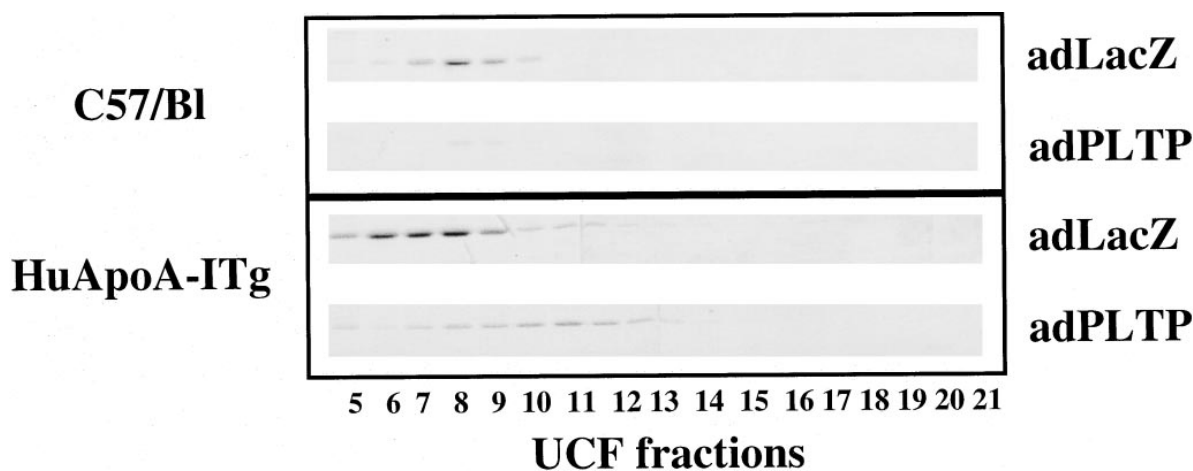


Fig. 3. Apoprotein A-I distribution in the ultracentrifugation fractions. The serum samples of the C57/Bl or HuApoA-ITg mice were collected on day 5 post injection of adPLTP or adLacZ, fractionated by density gradient ultracentrifugation, and analyzed in 4–20% SDS-PAGE. The proteins were visualized by Coomassie Brilliant Blue staining, and the identity of the apoA-I band was confirmed by Western blotting. The gradient fractions are indicated by numbers at the bottom. The density increases from right to left, and the fractions correspond to those shown in Fig. 2.

tein in the density range of LDL was apoA-I (Fig 3). As judged from Coomassie staining, the amount of apoB in these fractions was similar for the adLacZ and adPLTP-injected mice (data not shown). This suggests that the particles formed represent buoyant HDL and not LDL. The observation was further substantiated by nondenaturing native gradient gel electrophoresis which demonstrated a size increase in HDL particles (from the diameter of 10.8 nm to 11.4 nm) upon PLTP overexpression.


DISCUSSION

A number of in vitro studies have shown that PLTP, in addition to transferring phospholipids, facilitates the remodelling of HDL particle size distribution in the absence of other lipoproteins (8, 16, 18). However, the physiological role of plasma PLTP in lipoprotein metabolism is poorly understood. To provide an in vivo model for metabolic studies we used adenovirus-mediated gene transfer to produce mice that overexpress human PLTP. By this approach we were able to induce serum PLTP activities that were 2.5- to 4-fold compared to baseline levels. The initial PLTP activities measured in the wild-type (C57/Bl) mice prior to virus injection were similar to those reported by Albers et al. (20). However, in the human apoA-I transgenic mice, a higher basal PLTP activity than in wild-type mice was observed. This is somewhat contradictory to the results of Jiang et al. (19), and may be due to differences in HDL levels between the mice. The 2-fold increase in HDL we observed in the HuApoA-ITg mice may affect the baseline PLTP activity, as the protein has been reported to associate with HDL in the circulation (33, 34). Expression of human PLTP by the adenovirus vector in wild type (C57/Bl) mice resulted in a 4-fold increase in plasma PLTP activity on day 5. The overexpression of PLTP induced a drastic decrease in total serum cholesterol, triglycerides, and phospholipids. The prominent mouse plasma HDL fraction practically disappeared with a concomitant decrease in the amount of serum apoA-I. Further, an increase was observed in the free cholesterol/cholesteryl ester ratio in the mice. This may be due to the decreased LCAT activity levels observed in the mice that received adPLTP. The HuApoA-ITg mice showed a 2.5-fold increase in PLTP activity and a concurrent decrease in serum lipid values. The major difference to the wild type mice was that PLTP overexpression induced an increase of cholesterol in the LDL density interval accompanied by a drastic decrease in the HDL density range. The particles in the LDL interval contained apoA-I, and thus obviously represented larger buoyant HDL-like particles. Generation of large HDL particles has previously been reported upon in vitro incubation of HDL in the presence of human PLTP. The large particles showed a decreased level of apoA-I, and simultaneously with the formation of these particles, pre β -HDL-like particles were generated (16, 17, 35-37). The present work provides the first in vivo evidence for the PLTP-mediated enlargement of HDL particles.

The lipid/lipoprotein changes observed demonstrate

that abnormally high levels of PLTP drastically affect the mouse lipoprotein metabolism. There are at least two possible mechanisms that may, separately or in concert, account for the changes. First, the serum cholesterol esterification capacity may be reduced upon PLTP overexpression, which could lead to abnormal HDL particle composition and an increased turnover. The observation that the serum LCAT activity in adPLTP-injected mice was decreased supports this mechanism as one possible explanation for the reduced HDL levels. Second, PLTP-mediated HDL remodelling may lead to increased clearance of the resulting particles. As this work was submitted, Föger et al. (38) reported similar effects of adenovirus-mediated PLTP expression in wild-type mice. They also demonstrated that PLTP enhanced the uptake of phospholipids and cholesteryl ester from HDL by the liver. Our results clearly support these data and suggest that the large HDL particles formed after PLTP-mediated HDL conversion represent a population subject to rapid clearance.

It has been shown that during PLTP-mediated HDL conversion in vitro, apoA-I and phospholipids are shed from the surface of HDL particles, and these apoA-I-PL complexes have pre β -mobility (33). Generation of pre β -HDL was also demonstrated in the transgenic PLTP mice (19). The fact that in our experiments a relatively large amount of phospholipids and apoA-I was concentrated in the dense bottom fractions after density gradient ultracentrifugation indicates increased generation of similar apoA-I-PL-complexes.

At the moment we cannot conclusively pinpoint the mechanisms responsible for the lipoprotein changes observed. However, the present results give implications of the physiological importance of HDL conversion mediated by PLTP. We suggest that one of the major in vivo functions of PLTP is to facilitate the HDL remodelling to form large particles and apoA-I-PL-complexes. The large particles may be cleared from the circulation by the liver, whereas the small apoA-I-PL-complexes may infiltrate to the interstitial space and add to the pool of primary acceptors of peripheral cell membrane cholesterol in the process of reverse cholesterol transport. 

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