Increased Fecal Bile Acid Excretion in Transgenic Mice With Elevated Expression of Human Phospholipid Transfer Protein

Sabine M. Post, Rini de Crom, Rien van Haperen, Arie van Tol, Hans M.G. Princen

Objective—HDL plays a key role in protection against development of atherosclerosis by promoting reverse cholesterol transport from peripheral tissues to the liver for secretion into bile. Phospholipid transfer protein (PLTP) promotes the transfer of phospholipids between lipoproteins and modulates HDL size and composition, thereby having a crucial role in HDL metabolism. We investigated the effect of increased PLTP activity on removal of cholesterol from the body.

Methods and Results—On a chow diet, transgenic mice overexpressing human PLTP have a 15-fold increased plasma PLTP activity compared with wild-type mice (572.4±59.2 versus 38.6±3.6 μmol/mL per h). Plasma cholesterol, mainly present in HDL, is strongly decreased (−92%), caused by a rapid clearance from the circulation by the liver and leading to a 1.8-fold increase in hepatic cholesteryl esters. This results in a 2-fold increase in biliary acid secretion without changing the bile saturation index. Consequently, the transgenic mice show a 1.4-fold increase in the amount of excreted fecal bile acids compared with wild-type mice, whereas fecal neutral sterol excretion is unchanged.

Conclusions—Our data show that elevation of PLTP activity results in rapid disposal of cholesterol from the body via increased conversion into bile acids and subsequent excretion. (Arterioscler Thromb Vasc Biol. 2003;23:892-897.)

Key Words: bile acid • reverse cholesterol transport • HDL • phospholipid transfer protein • mouse

The level of plasma HDL cholesterol is inversely correlated with the risk of cardiovascular disease. HDL can remove cholesterol from the arterial wall and subsequently transport the lipid to the liver, where it can be converted into bile acids or secreted directly into the bile. This process is called reverse cholesterol transport. The conversion of cholesterol into bile acids and their subsequent fecal excretion is quantitatively the most important way for elimination of cholesterol from the body and the final step in reverse cholesterol transport. There are 2 major pathways involved in bile acid synthesis. The classical or neutral route in bile acid biosynthesis is initiated by 7α-hydroxylation of cholesterol catalyzed by the major rate-limiting enzyme cholesterol 7α-hydroxylase, which is located in the smooth endoplasmic reticulum. An alternative pathway in bile acid synthesis is the so-called acidic pathway initiated by the conversion of cholesterol by the enzyme sterol 27-hydroxylase, which is located in the inner mitochondrial membrane. The rate of bile acid synthesis is under control of different molecular mechanisms in which nuclear receptors are involved. In most species, the liver X-receptor α (LXRα) is involved in the feedforward regulation of cholesterol 7α-hydroxylase by cholesterol, whereas the feedback regulation by bile acids is mediated via the farnesoid X-receptor (FXR).

Phospholipid transfer protein (PLTP) plays a critical role in HDL metabolism. PLTP facilitates the transfer of phospholipids between lipoproteins and modulates HDL size and composition. Recent studies suggest that PLTP can have a potentially antiatherogenic role via its effect on HDL. It was shown that transgenic mice overexpressing human PLTP have an increased generation of pre-β-HDL, which is a very efficient acceptor of cholesterol from peripheral tissues. On the other hand, plasma levels of total HDL were decreased by an accelerated catabolism of HDL particles. Recently, an unexpected cellular role of PLTP was found in PLTP knockout mice, in which it was demonstrated that PLTP can regulate the secretion of apolipoprotein-B-containing lipoproteins. PLTP deficiency not only resulted in a reduced production and plasma levels of apolipoprotein-B-containing lipoproteins but also in increased antioxidant potential and in markedly decreased atherosclerosis. Therefore, increased PLTP expression seems to have various effects on lipoprotein metabolism.

In the present study, we investigated the metabolic fate of HDL cholesterol in transgenic mice overexpressing human PLTP by studying the way it is handled by the liver. Our data demonstrate that elevation of PLTP activity results in a rapid disposal of cholesterol from the body via increased bile acid

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excretion, suggesting an enhanced HDL-mediated reverse cholesterol transport.

Methods

Animals
HuPLTP-overexpressing mice on a C57Bl/6 background (line A2) and wild-type C57Bl/6 as controls were used.17,18 The experiments were performed with adult male mice, which were maintained on 12-hour dark and 12-hour light cycles and allowed free access to chow and water. Body weight of the mice and the consumption of diet and water were recorded weekly and did not differ during the whole experimental period. Liver mass of the PLTP overexpressing mice was the same as that of the wild-type mice. Institutional guidelines for animal care were observed in all experiments.

Assay of Plasma PLTP Activities
Plasma PLTP activity was measured using a liposome vesicles-HDL system, as described.12 In short, plasma samples were incubated with [3H]phosphatidylcholine–labeled liposomes and an excess of pooled normal HDL for 45 minutes at 37°C, followed by precipitation of the lipoproteins with a mixture of NaCl, MgCl2, and heparin (final concentrations, 230 mmol/L, 92 mmol/L, and 200 U/mL, respectively). After centrifugation, the radioactivity transferred to HDL was counted in the supernatant. Plasma was diluted 50-fold (wild-type mice) or 500-fold (transgenic mice) before assay, and the measured PLTP activities were linearly correlated with the amount of diluted plasma (12.5 to 25 μL) used in the incubations. All assays were performed in duplicate, using the same batch of substrates.

Measurement of Serum Lipids and Lipoproteins
Total cholesterol, triglycerides (corrected for plasma levels of free glycerol), phospholipids, and free fatty acids were measured enzymatically (CHOD-PAP method, Boehringer Mannheim, No. 236691; GPO-trinder, Sigma, No. 337-B; and PAP150, BioMérieux and No. 990-54009 Wako Chemicals GmbH, NEFA-C kit Wako Chemicals GmbH, respectively). Lipoprotein profiles were measured by gel filtration from pooled plasma of 6 mice, as described previously.13 Ketone bodies were measured with β-hydroxybutyrate kit (Sigma No. 310).

Determination of Lipoprotein Lipase and Hepatic Lipase Activity
Postheparin plasma from fasted mice (4 hours) was collected from the retro-orbital plexus 20 minutes after injection of heparin (1.0 U/g body weight). The assay of postheparin plasma-lipolytic activity was performed in the presence or absence of 1 mol/L NaCl to estimate both the lipoprotein lipase (LPL) and hepatic lipase (HL) activity. LPL activity was calculated as the portion of total lipase activity inhibited by 1 mol/L NaCl,19

Enzyme Activities and mRNA Levels of Cholesterol 7α-Hydroxylase, Sterol 27-Hydroxylase, and HMG-CoA-Reductase and Measurement of Liver Lipids
Enzymatic activities of cholesterol 7α-hydroxylase and sterol 27-hydroxylase in mouse liver microsomes and mitochondria, respectively, were determined as described previously,20 measuring the mass conversion of cholesterol into 7α- and 27-hydroxycholesterol. HMG-CoA-reductase activity in freshly isolated liver microsomes21 and liver lipids20,22 after lipid extraction23 were measured as described.

Isolation of total RNA and subsequent electrophoresis, Northern blotting, probes, and hybridization techniques were performed as described previously.20,24,25 The GAPDH mRNA or 18S rRNA was used as an internal standard to correct for differences in the amount of total RNA applied onto the gel. mRNA levels were quantitated as described previously.20

Determination of Concentration and Composition of Bile Acids and Neutral Sterols in Feces
Fecal production during a 3-day period was separated from the wood shavings. Fecal samples were lyophilized and weighed. For extraction of bile acids, an aliquot of dried feces (5 mg) was incubated in 1 mL alkaline methanol (methanol: 1 mol/L NaOH 3:1 [vol/vol]) for 2 hours at 80°C in screw-capped tubes using nor-hyodeoxycholate as an internal standard. Then 9 mL of distilled water was added, and the tubes were mixed and centrifuged. The supernatant was subsequently applied to a prepared Sep-Pak C18 solid-phase extraction cartridge.26 After a clean up by wash procedures, bile acids were eluted with 75% methanol. The solvent was evaporated to dryness, and the bile acids were derivatized by incubation with 50 μL trifluoroacetic anhydride and 30 μL 1,1,1,3,3,3-hexafluoro-2-propanol for 1 hour at 60°C. The bile acid derivatives were separated on a 25 m×0.25 mm capillary GC column (CP Sil 5B, Chrompack International) in a 3800 GC gas chromatograph (Varian) equipped with a flame ionization detector. The injector and the flame ionization detector were kept at 300°C. The column temperature was programmed from 230 to 280°C. Bile acid derivatives were introduced by split injection (split ratio, 20:1). Quantitation was based on the area ratio of the individual bile acid to the internal standard.

To extract neutral steroids from feces, dried feces (10 mg) were treated with 1 mL alkaline methanol as described for bile acid measurement using 5α-cholostane as internal standard. After treatment, the tubes were cooled to room temperature and the neutral steroids were extracted 3 times with 3 mL petroleum ether. The combined petroleum ether layers were evaporated to dryness, and the neutral steroids were silylated by DMS–Silprep (Alltech). Analysis of the sterol derivatives was performed by GC, applying the same column as for the bile acids. Quantitation was based on the area ratio of the individual neutral steroid to the internal standard 5α-cholostane.

Determination of Biliary Lipid Secretion
Separate groups of animals were used for these experiments. The common bile duct of anesthetized wild-type and HuPLTP-overexpressing mice was ligated, and the gallbladder was cannulated. Bile was collected in 15-minute intervals for 105 minutes. Cholesterol and phospholipid in bile were determined after lipid extraction,23 as described previously.22 Total bile acid in bile was determined using a commercial kit from Nycomed. The biliary bile acid composition was determined by gas liquid chromatography.20 The bile acid pool size was determined from the area under the curve during the first 90 minutes of bile collection.

Statistics
Data were analyzed statistically using the nonparametric Mann–Whitney test with the level of significance selected to be P=0.05. Values are expressed as mean±SD.

Results

Effect of HuPLTP Overexpression on Plasma and Hepatic Lipid Levels
PLTP activity was measured in plasma samples of HuPLTP-overexpressing and wild-type mice. In the PLTP-overexpressing mice, the PLTP activity was 15-fold higher compared with wild-type mice (Table 1). The increase in PLTP activity resulted in dramatically reduced total plasma cholesterol levels (−92%), mainly in the HDL fraction (Figure 1). The amounts of phospholipids and triglycerides in plasma were also decreased (Table 1), whereas plasma free fatty acids as well as ketone bodies did not change (data not shown). Both lipoprotein lipase activity (4.01±1.41 versus 1.90±1.42 μmol FFA/mL per h, P=0.02) as well as hepatic lipase activity (1.88±0.73 versus 0.95±0.34 μmol FFA/mL per h, P=0.04).
To investigate changes in hepatic lipid content, we measured free cholesterol, cholesteryl esters, triglycerides, and phospholipids in the livers of wild-type and HuPLTP-overexpressing mice (Table 2). Whereas free cholesterol levels were not changed, cholesteryl ester content in the overexpressing mice was 1.8-fold higher compared with the wild-type mice. The hepatic triglyceride content in the PLTP-overexpressing mice did not change significantly (Table 2), whereas phospholipids decreased significantly (−22%).

Bile Analyses
To investigate the metabolic fate of hepatic cholesterol, we measured bile flow and biliary output of cholesterol and bile acids in wild-type and HuPLTP-overexpressing mice. As shown in Table 3, bile flow is significantly higher in the HuPLTP-overexpressing mice. In addition, bile acid and cholesteryl output were both significantly enhanced (2.0- and 2.5-fold, respectively) in the HuPLTP-overexpressing mice. The bile acids present in the bile were predominantly cholate (52%) and muricholate (α, β, ω) (41%) and did not differ between the groups. In addition to a higher bile acid output, the bile acid pool size, as determined from the washout curves, was 2.4-fold larger in the HuPLTP transgenic mice compared with the wild-type mice (100±40% versus 237±53%; P<0.05). Next to this, phospholipid output was also 1.7-fold higher in HuPLTP transgenic mice compared with the wild-type mice. Because excretion of all biliary lipids was elevated, the changes in biliary lipid output did not have an effect on the lithogenicity index of the bile (1.19±0.44 versus 1.41±0.18; n=6).

Fecal Cholesterol and Bile Acids, Hepatic Enzyme Activities, and mRNA Levels
To investigate whether the increased biliary bile acid and cholesterol excretion resulted in an enhanced fecal sterol output, we determined the bile acid and neutral sterol content in the feces of wild-type and HuPLTP-overexpressing mice. As shown in Figure 2, fecal neutral sterol excretion did not change, whereas fecal bile acid excretion was significantly increased (1.4-fold), which reflects an increased bile acid synthesis. The fecal bile acid composition was not different in the HuPLTP-overexpressing mice compared with the wild-type mice. The bile acids present in the feces were predominantly deoxycholate (40%), muricholate (α, β, ω) (39%), and cholate (10%), with minor amounts of lithocholate, chenodeoxycholate, hyodeoxycholate, and ursodeoxycholate.

The increase in bile acid synthesis did not result from an induction in the expression of cholesterol 7α-hydroxylase or sterol 27-hydroxylase, because we could not detect any significant changes in the enzyme activity and mRNA levels of these bile acid synthetic enzymes (Table 4).

We additionally examined whether the increased expression of PLTP and the subsequently enhanced hepatic cholesteryl ester content and bile acid synthesis resulted in a change in cholesterol synthesis. HMG-CoA reductase activity measured in liver microsomes was significantly lower (−32%) in the PLTP-overexpressing mice compared with the wild-type mice (Table 4).

Discussion
In this study, we showed that hepatic overexpression of human PLTP in mice results in enhanced bile acid synthesis and increased fecal bile acid excretion.

Overexpression of human PLTP in mice either by a transgene or via adenoviral-mediated gene transfer leads to a decrease in plasma HDL concentration.12–14 The decrease in plasma HDL levels is caused by an accelerated clearance of HDL cholesteryl esters by the liver13 and leads to elevated levels of cholesteryl esters in the liver of PLTP transgenic mice, as shown in the present study. The metabolic fate of this enlarged amount of hepatic cholesteryl esters is unknown and subject of the present study. The elevated level of cholesteryl esters in the liver resulted in an expected downregulation of the hepatic cholesterol synthesis, as reflected by a reduction in HMG-CoA-reductase activity. This can also contribute to the decrease in plasma cholesterol levels found in the PLTP transgenic mice.

### Table 1. Plasma Lipid Levels and PLTP Activity

<table>
<thead>
<tr>
<th></th>
<th>Phospholipids, mmol/L</th>
<th>Cholesterol, mmol/L</th>
<th>Triglycerides, mmol/L</th>
<th>PLTP Activity, μmol/mL per h</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type</td>
<td>1.39±0.44</td>
<td>1.63±0.30</td>
<td>0.23±0.10</td>
<td>38.6±3.6</td>
</tr>
<tr>
<td>HuPLTP</td>
<td>0.46±0.28† (30)</td>
<td>0.23±0.18‡ (8)</td>
<td>0.06±0.02* (26)</td>
<td>572.4±59.2† (1483)</td>
</tr>
</tbody>
</table>

Data are mean±SD (n=6 mice per group). Values in parentheses are percentages of the wild-type values.

A significant difference is indicated by an asterisk (*P<0.01, †P<0.001, ‡P<0.0001).
TABLE 2. Hepatic Lipid Levels

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>HuPLTP</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>FC, µg/mg protein</td>
<td>CE, µg/mg protein</td>
</tr>
<tr>
<td>Wild-type</td>
<td>8.98±0.40</td>
<td>3.35±0.73</td>
</tr>
<tr>
<td>HuPLTP</td>
<td>8.43±0.60 (94)</td>
<td>6.15±1.29* (184)</td>
</tr>
</tbody>
</table>

Data are mean±SD (n=6 mice per group). Values in parentheses are percentages of the wild-type values.

It is well-known that elevated levels of hepatic cholesterol can induce the expression of cholesterol 7α-hydroxylase in different animal models via LXRα-mediated gene transcription. However, the relatively modest increase in hepatic cholesterol ester in the PLTP transgenic mice did not result in enhanced enzyme activities nor in elevated mRNA levels of cholesterol 7α-hydroxylase and sterol 27-hydroxylase. Possibly, the amount of regulatory cholesterol metabolites (oxysterols) remains too low to induce cholesterol 7α-hydroxylase expression. It should be noted that although there was an increase in hepatic cholesterol levels, the absolute cholesterol content is still low compared with cholesterol-fed mice or rats. In the latter experiments, hepatic cholesterol ester levels were 8- to 13-fold increased, compared with an

TABLE 3. Bile Flow and Biliary Lipid Secretion

<table>
<thead>
<tr>
<th></th>
<th>Wild-type</th>
<th>HuPLTP</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow, µL/min/100 g BW</td>
<td>6.62±1.48</td>
<td>8.69±0.83* (132)</td>
</tr>
<tr>
<td>Bile acid output, nmol/min/100 g BW</td>
<td>197±116</td>
<td>401±112* (204)</td>
</tr>
<tr>
<td>Cholesterol output, nmol/min/100 g BW</td>
<td>11.7±3.1</td>
<td>29.0±9.3* (247)</td>
</tr>
<tr>
<td>Phospholipid output, nmol/min/100 g BW</td>
<td>40.0±13.0</td>
<td>66.8±13.8* (167)</td>
</tr>
</tbody>
</table>

Data are mean±SD (n=6 mice per group). Values in parentheses are percentages of the values in wild-type mice. BW indicates body weight.

A significant difference is indicated by an asterisk (*P<0.05).

This increase in biliary secretion of bile acids and cholesterol could be attributable to an increased expression of the transporter genes BSEP, ABCG5, and ABCG8, which have been shown to be upregulated by FXR and LXRα as a consequence of a higher pool of bile acids and oxysterols in the transgenic mice. Consequently, the elevated biliary cholesterol output did not result in an increased lithogenicity index, which is an important indicator of bile stone formation.

In the present study, only transgenic mice with relatively high expression of PLTP were studied. However, in a series of transgenic mice with increasing PLTP expression, a PLTP dose-dependent relation was found with both plasma HDL levels and atherosclerosis susceptibility, suggesting that at least these phenotypic effects are not restricted to A2 line. In PLTP mice with 4.5-fold elevated PLTP activity (lines P4 and A3), we also found an increased elevated fecal bile acid output (1.2-fold). Putting all of the data together from mice from the 4 PLTP transgenic lines published before, a statistically significant positive correlation (R²=0.42; P<0.0005) was observed between PLTP expression and fecal bile acid output (results not shown), indicating that the effects described in this study are not restricted to the line of transgenic mice used.

In contrast to the PLTP-overexpressing mice, other mouse models in which the HDL-mediated uptake of cholesterol by...
the liver was stimulated did not show changes in bile acid synthesis. For example, hepatic overexpression of the HDL-receptor SR-BI in mice resulted in an increased biliary cholesterol excretion but not in an elevated bile acid output.31,32 Similarly, adenosine-mediated gene transfer of the recently identified cholesterol and phospholipid transporter ABCG1 led to an increased reverse cholesterol transport and subsequently to an enhanced biliary cholesterol secretion without affecting biliary bile acid secretion.33 A drawback of an increase in biliary cholesterol without a simultaneous elevation of biliary bile acid excretion is the negative effect on cholesterol gallstone formation and the adverse changes in lipoprotein metabolism, because biliary cholesterol can be taken up again in the intestine. In contrast, when cholesterol is converted into bile acids, there will be a proportionally increased fecal excretion of bile acids, resulting in the net loss of cholesterol from the body. The observed increase in biliary lipid secretion resulted in an enhanced fecal bile acid excretion. However, we did not find a change in the amount of fecal neutral sterols. Because bile acids play an important role in the solubilization of cholesterol, the elevated amount of bile acids in the intestine may consequently increase cholesterol absorption, resulting in unchanged fecal neutral sterol secretion.

Recently it was found that PLTP deficiency in mice results in reduced production and levels of apolipoprotein B—containing lipoproteins and markedly decreased atherosclerosis,15 indicating that, next to an effect in plasma, PLTP can exert an effect at the cellular level. We recently found a modest increase (<1.5-fold) of VLDL secretion in PLTP-overexpressing mice.18,34 In the present study, however, we found strongly decreased levels of plasma triglycerides in the PLTP-overexpressing mice. This is in agreement with studies in which adenosine-mediated overexpression of PLTP was studied.13,14 Thus, high levels of PLTP activity seem to result in a decrease in plasma triglycerides. In addition, the HuPLTP-overexpressing mice have reduced LPL and HL activity and show no difference in ketone bodies, which are a marker of hepatic β-oxidation. These results suggest that elevated PLTP expression causes remodeling of lipoproteins and results in whole particle uptake of triglycerides-rich lipoproteins as well as enlarged HDL particles.13,14

Whether increases in expression of PLTP in humans also will lead to an increased removal of cholesterol from the body is not known. Recently, Eriksson et al35 showed that HDL elevation by infusion of recombinant proapolipoprotein A-I in humans and subsequent enhancement of reverse cholesterol transport resulted in an increased fecal steroid excretion, indicating that modulating HDL metabolism in humans may be a useful way to remove cholesterol, ie, in the form of bile acids and cholesterol.

We recently found that elevated plasma PLTP activity results in increased susceptibility of diet-induced atherosclerosis in heterozygous LDL-receptor knockout mice, probably caused by a decrease in plasma HDL.18 However, PLTP also has antiatherogenic properties, as revealed by increased fecal bile acid secretion described in the present study. In addition, we previously reported that elevated PLTP leads to an increase in the formation of pre-β-HDL, which is a very efficient receptor of cellular cholesterol.12 Thus, PLTP seems to have atherogenic and antiatherogenic properties. Although the atherogenic potential apparently dominates in heterozygous LDL-receptor knockout mice fed a high-fat, high-cholesterol diet, this is not necessarily true for other animal models of atherosclerosis or humans. Additional studies are needed to unravel the exact mechanism by which elevated PLTP activity affects the development of atherosclerosis.

The high level of PLTP expression used in the present study resulted in higher plasma PLTP activity than that observed in men. However, atherosclerosis in humans is a lengthy process in which a more modest elevation of PLTP activity could have significant long-term effects. Recent studies indicate that LXR activation leads to increased expression of PLTP.36 However, PLTP activity was only measured by Cao et al,36 who found a modest increase at very high doses of the LXR agonist T0901317,37,38 In addition, treatment with LXR agonist affects the expression of several genes involved in lipid metabolism and may result in a large increase in liver triglycerides.38 Some of the reported effects on HDL metabolism also occur in PLTP-deficient mice, indicating that they are not dependent on PLTP activity.36 Thus, treatment with LXR agonists will have other effects in addition to an increase of PLTP activity. Future studies will have to establish whether modulation of PLTP activity is feasible to achieve enhanced bile acid excretion in humans.

Acknowledgments
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References

<table>
<thead>
<tr>
<th>TABLE 4. Hepatic Enzyme Activities and mRNA Levels</th>
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<tr>
<td>Enzyme activity, nmol/mg per h</td>
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<tr>
<td>Wild-type</td>
</tr>
<tr>
<td>7α-hydroxylase</td>
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<tr>
<td>27-hydroxylase</td>
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<tr>
<td>HMG-CoA reductase mRNA levels, % of control</td>
</tr>
<tr>
<td>7α-hydroxylase</td>
</tr>
<tr>
<td>27-hydroxylase</td>
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</tbody>
</table>

Data are mean ± SD (n=6 mice per group). Values in parentheses are percentages of the values in wild-type mice.

Enzyme activities and mRNA levels were measured in wild-type and HuPLTP overexpressing mice fed a chow diet.

A significant difference is indicated by an asterisk (*P<0.0001).
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