Secretory phospholipase A_2 increases SR-BI-mediated selective uptake from HDL but not biliary cholesterol secretion

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Abstract **High density lipoprotein cholesterol represents a major source of biliary cholesterol. Secretory phospholipase A_2 ($sPLA_2$) is an acute phase enzyme mediating decreased plasma HDL cholesterol levels. Clinical studies reported a link between increased $sPLA_2$ expression and the presence of cholesterol gallstones. The aim of our study was to investigate whether the overexpression of human $sPLA_2$ in transgenic mice affects biliary cholesterol secretion and gallstone formation. Liver weight ($P < 0.01$) and hepatic cholesterol content ($P < 0.01$) were significantly increased in $sPLA_2$ transgenic mice compared with controls as a result of increased scavenger receptor class B type I (SR-BI)-mediated hepatic selective uptake of HDL cholesterol ($P < 0.01$), whereas hepatic SR-BI expression remained unchanged. However, biliary cholesterol secretion as well as fecal neutral sterol and fecal bile salt excretion remained unchanged in $sPLA_2$ transgenic mice. Furthermore, gallstone prevalence in response to a lithogenic diet was identical in both groups. These data demonstrate that *i*) increased flux of cholesterol from HDL into the liver via SR-BI as a result of phospholipase modification of the HDL particle translates neither into increased biliary and fecal sterol output nor into increased gallstone formation, and *ii*) increased $sPLA_2$ expression in patients with cholesterol gallstones might be a consequence rather than the underlying cause of the disease.**—Tietge, U. J. F., N. Nijstad, R. Havinga, J. F. W. Baller, F. H. van der Sluijs, V. W. Bloks, T. Gautier, and F. Kuipers. **Secretory phospholipase A_2 increases SR-BI-mediated selective uptake from HDL but not biliary cholesterol secretion.** ***J. Lipid Res.*** **2008.** 49: **563–571.**

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The protective effects of HDL against atherosclerotic cardiovascular disease are generally ascribed to the central role of the HDL particle in reverse cholesterol transport (RCT) (1, 2). RCT in this context comprises the ability of HDL to mediate the transport of excess cholesterol from lipid-laden macrophages within the vascular wall back to the liver for excretion into the bile, which represents the major route for irreversible removal of cholesterol from the body.

The acute phase protein secretory group IIA phospholipase A_2 ($sPLA_2$) is a low molecular mass (14 kDa) enzyme with exclusive phospholipase activity hydrolyzing phospholipids at the *sn*-2 position (3, 4). Compared with wild-type C57BL/6 controls that lack the endogenous mouse $sPLA_2$ enzyme as a result of a frameshift mutation (5), human $sPLA_2$ transgenic mice have significantly decreased plasma HDL cholesterol levels, suggesting that $sPLA_2$ is a major mediator of the decreased plasma HDL cholesterol invariably associated with acute as well as chronic inflammatory states (6–8). Patients with chronic inflammatory diseases have a higher risk of developing atherosclerotic cardiovascular disease (9, 10), and $sPLA_2$ transgenic mice develop atherosclerotic lesions even on a chow diet (11). On the other hand, atherosclerosis itself is increasingly recognized as a localized inflammatory condition within the vessel wall (12, 13). The pathophysiological significance of $sPLA_2$ in this respect is underlined by studies demonstrating increased circulating plasma levels of $sPLA_2$ to be predictive for acute coronary events (14–16).

The scavenger receptor class B type I (SR-BI) has been identified as a key component in RCT mediating the selective uptake of HDL cholesterol by the liver for subsequent biliary excretion (17, 18). SR-BI knockout mice and mice with attenuated hepatic SR-BI expression have increased plasma HDL cholesterol levels and decreased biliary cholesterol levels (19, 20). In contrast, hepatic overexpression of SR-BI results in decreased plasma HDL

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cholesterol levels and increased biliary cholesterol excretion in response to acute overexpression (18) and also in a transgenic mouse model (20). Hepatic SR-BI overexpression is protective against atherosclerosis despite low HDL cholesterol plasma levels acutely after adenovirus-mediated gene transfer (21) and also using transgenic mice (22). These studies suggest that the flux of cholesterol from HDL into the liver via SR-BI might reflect functional RCT more accurately than mere steady-state HDL cholesterol plasma levels. $sPLA_2$ modification of the HDL particle stimulates selective uptake into the liver and the adrenals in vivo (23). These data indicate that $sPLA_2$ expression might affect hepatic cholesterol metabolism.

Therefore, the aim of the present study was to investigate the consequences of $sPLA_2$-mediated HDL phospholipid hydrolysis on hepatic cholesterol metabolism. Our data demonstrate increased SR-BI-mediated cholesterol flux from HDL into the liver in human $sPLA_2$ transgenic mice, resulting in hepatic cholesterol storage. However, neither biliary cholesterol secretion nor fecal sterol excretion were affected under these conditions. These results indicate that increased influx through SR-BI into the liver might not be sufficient to enhance mass RCT. These data might have important implications for the concept of RCT and the role of hepatic cholesterol metabolism therein.

MATERIALS AND METHODS

Animals

C57BL/6J control mice were obtained from Charles River (Sulzfeld, Germany). The transgenic mice expressing human group IIA $sPLA_2$ driven by its endogenous promoter backcrossed to the C57BL/6J genetic background for >14 generations have been described previously (6, 24). The animals were caged in animal rooms with alternating 12 h periods of light (from 7:00 AM to 7:00 PM) and dark (from 7:00 PM to 7:00 AM), with ad libitum access to water and mouse chow diet. Animal experiments were performed in accordance with national laws. All protocols were approved by the responsible ethics committee of the Landesamt für Gesundheit, Ernährung, und Technische Sicherheit Berlin.

Plasma lipid and lipoprotein analysis

Mice (n = 8 per group) were bled from the retro-orbital plexus after a 4 h fast using heparinized capillary tubes. Aliquots of plasma were stored at −20°C until analysis. Plasma total cholesterol, triglycerides, and phospholipids were measured enzymatically using commercially available reagents (Wako Pure Chemical Industries, Neuss, Germany). HDL cholesterol levels were determined after precipitation of apolipoprotein B-containing lipoproteins using phosphotungstic acid-$MgCl_2$ (Sigma Diagnostics, St. Louis, MO).

HDL kinetic studies

Autologous apolipoprotein E-free HDL isolated by sequential ultracentrifugation ($1.063 < d < 1.21$) was prepared from pooled mouse plasma and labeled with ^{125}I-tyramine-cellobiose and cholesteryl hexadecyl ether (cholesteryl-1,2-^{3}H; New England Nuclear Life Sciences Products) essentially as described (23), and 1 μCi of the ^{125}I-tyramine-cellobiose-HDL and 1 million dpm of the [^{3}H]cholesteryl ether (CE)-HDL were injected via the tail vein. Plasma decay curves for both tracers were generated by dividing the plasma radioactivity at each time point by the radioactivity at the initial 5 min time point after tracer injection. Fractional catabolic rates (FCRs) were determined from the area under the plasma disappearance curves fitted to a bicompartmental model by use of the SAAM II program (23). Organ uptake of HDL apolipoproteins (^{125}I) and ^{3}H-HDL-CEs was determined and expressed as a percentage of the injected dose calculated by multiplying the initial plasma counts (5 min time point) with the estimated plasma volume (3.5% of total body weight). Selective uptake into organs was determined by subtracting the percentage of the injected dose of ^{125}I-HDL recovered in each organ from the percentage of the injected dose of [^{3}H]HDL CE and correcting the value for tissue weight. The hepatic uptake rate was calculated as the product of the relative tissue FCR and the HDL CE plasma pool, whereby the relative tissue FCR is represented by the product of plasma FCR and the percentage of initial plasma counts recovered in each organ. The HDL CE plasma pool was determined by multiplying the plasma volume of each mouse with the respective HDL CE concentration.

Cell culture and HDL selective uptake experiments

LdlA cells lacking LDL receptor expression and ldlA cells stably transfected with a murine SR-BI cDNA (ldlA[mSR-BI]) were kindly provided by Dr. Monty Krieger (Massachusetts Institute of Technology, Boston, MA) and cultured as described (17). For HDL uptake experiments, 5% lipoprotein-depleted serum was used. A total of 10 μg/ml ^{125}I/[^{3}H]CE HDL isolated and radioactively labeled as described above was added to the cells. After a 5 h incubation, the cells were washed three times with PBS (pH 7.4) and lysed with 0.5 ml of 0.1 M NaOH. Tracer uptake was calculated as the counts recovered from the cells as a percentage of the total dose (counts from cells added to the counts from medium). Selective HDL CE uptake was determined by subtracting the percentage of the total dose of ^{125}I recovered from the cells from the percentage of the total dose of ^{3}H recovered from the cells.

Analysis of liver lipid composition

The hepatic content of total and free cholesterol, phospholipids, and triglycerides was measured as described (25). Protein concentrations in liver homogenates were determined using the BCA assay kit (Pierce).

Bile collection and assessment of biliary excretion of cholesterol, phospholipids, and bile acids

Bile was collected by cannulation of the gallbladder under Hypnorm (fentanyl/fluanisone; 1 ml/kg) and diazepam (10 mg/kg) anesthesia, using a humidified incubator to maintain body temperature. Bile collection was performed for 40 min, and production was determined gravimetrically. Biliary bile salt, cholesterol, and phospholipid concentrations were determined and the respective biliary excretion rates calculated as described previously (25).

Gallstone formation experiments

To investigate gallstone formation, groups of $sPLA_2$ transgenic mice (n = 12) and C57BL/6 controls (n = 15) were fed a lithogenic diet containing 1.25% cholesterol, 15% total fat, and 0.5% cholic acid (TD90221; Harlan Teklad, Madison, WI) for 5 weeks. At the end of this period, the formation of macroscopic gallstones was assessed in fresh gallbladder bile. In separate experiments, the effect of the lithogenic diet on bile flow and biliary

cholesterol secretion was determined in the respective experimental groups essentially as described above.

Analysis of gene expression by real-time quantitative PCR

Total RNA from mouse livers was isolated using Trizol (Invitrogen) and quantified with Ribogreen (Molecular Probes, Inc., Eugene, OR). cDNA synthesis was performed from 2 μg of total RNA using reagents from Applied Biosystems (Darmstadt, Germany). Real-time quantitative PCR was carried out using an ABI-Prism 7700 (Applied Biosystems) sequence detector with the default settings. PCR primers and fluorogenic probes were designed with the Primer Express Software (Applied Biosystems) and synthesized by Eurogentec (Seraing, Belgium). The mRNA expression levels presented were calculated relative to the average of the housekeeping gene cyclophilin and further normalized to the relative expression levels of the respective controls.

Isolation of liver plasma membranes and Western blot for SR-BI

Liver plasma membranes were isolated using livers pooled from three mice essentially as described previously (26). The final membrane pellets were resuspended in buffer containing 250 mM sucrose and 10 mM Tris, pH 7.4, homogenized by 50 strokes through a Dounce homogenizer, and immediately stored at −80°C for further analysis. Protein concentrations were determined using the BCA reagents (Pierce). Relative enrichments of Na^+/K^+-ATPase and Mg^{2+}-ATPase as marker enzymes for plasma membrane fractions were measured on an EL 808 Ultra Microplate Reader (Bio-Tek, Winooski, VT) to determine the purity of the isolated membranes in the different preparations. For Western blot analysis, protein samples from liver homogenates (total cellular SR-BI content) and the plasma membrane fractions (plasma membrane-associated SR-BI) were resolved on 10% SDS-PAGE gels. mSR-BI was visualized using a polyclonal rabbit anti-mSR-BI primary antibody (Novus Biologicals, Littleton, CO), followed by the appropriate secondary antibody.

Fecal sterol analysis

Mice were housed individually, and feces was collected over a period of 2 days. Fecal samples were lyophilized and weighed. Aliquots thereof were used for the determination of neutral and acidic sterol content by gas-liquid chromatography as described (25).

Statistical analysis

Statistical analyses were performed using the Statistical Package for the Social Sciences (SPSS, Inc., Chicago, IL). Data are presented as means ± SEM. Statistical analysis was performed using the Mann-Whitney U-test to compare different groups. Statistical significance for all comparisons was assigned at $P < 0.05$.

RESULTS

$sPLA_2$ expression in transgenic mice results in decreased plasma HDL cholesterol levels and increased hepatic cholesterol content

Compared with wild-type controls, total cholesterol levels in $sPLA_2$ transgenic mice were decreased significantly (76 ± 4 vs. 95 ± 5 mg/dl; $P < 0.01$, n = 8), mainly as a result of decreased plasma HDL cholesterol levels (49 ± 3 vs. 74 ± 4 mg/dl; $P < 0.01$, n = 8). Plasma phospholipids were also decreased significantly in $sPLA_2$ transgenic mice (128 ± 9 vs. 192 ± 5 mg/dl; $P < 0.01$, n = 8), whereas plasma triglycerides were not different between $sPLA_2$ transgenic and wild-type control animals (60 ± 10 vs. 54 ± 7 mg/dl; $P < 0.01$, n = 8). These data are consistent with previous results (6).

Liver weight was increased significantly in $sPLA_2$ transgenic mice compared with controls (1.70 ± 0.06 vs. 1.41 ± 0.06 g; $P < 0.01$). Hepatic cholesterol content per gram of liver (6.23 ± 0.26 vs. 5.50 ± 0.13 μmol/g; $P < 0.05$) (**Table 1**) as well as total hepatic cholesterol content (10.26 ± 0.55 vs. 7.77 ± 0.41 μmol/liver; $P < 0.01$) (Table 1) were significantly higher in $sPLA_2$ transgenic mice compared with controls, which was entirely attributable to an increase in free cholesterol ($P < 0.001$) (Table 1), whereas hepatic cholesteryl ester content did not differ between groups (Table 1). Hepatic triglyceride concentrations were decreased in $sPLA_2$ transgenic mice (8.7 ± 0.6 vs. 16.3 ± 3.3 μmol/g; $P < 0.05$) (Table 1), whereas hepatic phospholipid concentrations did not differ between the experimental groups of mice (Table 1).

SR-BI-mediated selective uptake of HDL cholesteryl ester is increased by $sPLA_2$-mediated modification of the particle in vivo and in vitro

To elucidate the metabolic basis of decreased plasma HDL cholesterol levels and increased hepatic cholesterol content in $sPLA_2$ transgenic mice, we performed HDL kinetic studies with autologous HDL. Fractional catabolic rates for HDL apolipoproteins (0.212 ± 0.021 vs. 0.085 ± 0.004 pools/h; $P < 0.001$) as well as HDL CE (0.238 ± 0.013 vs. 0.144 ± 0.018 pools/h; $P < 0.001$) were significantly higher in $sPLA_2$ transgenic mice compared with controls (see supplementary Fig. IA, IB).

In vivo, $sPLA_2$-mediated modification of HDL particles resulted in a significant increase of selective uptake into organs with high expression of SR-BI, namely the liver (30 ± 2 vs. 41 ± 3%/μg organ; $P < 0.01$) (**Fig. 1A**) and the adrenals (57 ± 7 vs. 89 ± 10%/μg organ; $P < 0.001$) (Fig. 1A). The mass HDL CE flux into livers of $sPLA_2$ transgenic mice was increased significantly compared with that in controls (44 ± 3 vs. 68 ± 5 μg/h/liver; $P < 0.01$) (Fig. 1B). HDL apolipoprotein catabolism by the kidneys

TABLE 1. Hepatic lipid composition in $sPLA_2$ transgenic mice and wild-type C57BL/6 littermates

Variable	Units	C57BL/6 (n = 8)	Human $sPLA_2$ Transgenic (n = 8)
Total cholesterol	μmol/g	5.50 ± 0.13	6.23 ± 0.26[a]
	μmol/liver	7.77 ± 0.41	10.26 ± 0.55[a]
Free cholesterol	μmol/g	4.03 ± 0.15	4.83 ± 0.21[a]
	μmol/liver	2.08 ± 0.16	2.30 ± 0.14[a]
Cholesteryl ester	μmol/g	1.47 ± 0.08	1.41 ± 0.09
	μmol/liver	5.69 ± 0.30	7.96 ± 0.46
Phospholipids	μmol/g	33.8 ± 1.1	35.8 ± 1.1
	μmol/liver	47.7 ± 2.4	59.0 ± 2.9[a]
Triglycerides	μmol/g	16.3 ± 3.3	8.7 ± 0.6[a]
	μmol/liver	23.5 ± 5.0	14.5 ± 1.4

$sPLA_2$, secretory phospholipase A_2. Values shown are means ± SEM.
[a] Significantly different from wild-type mice (at least $P < 0.05$).

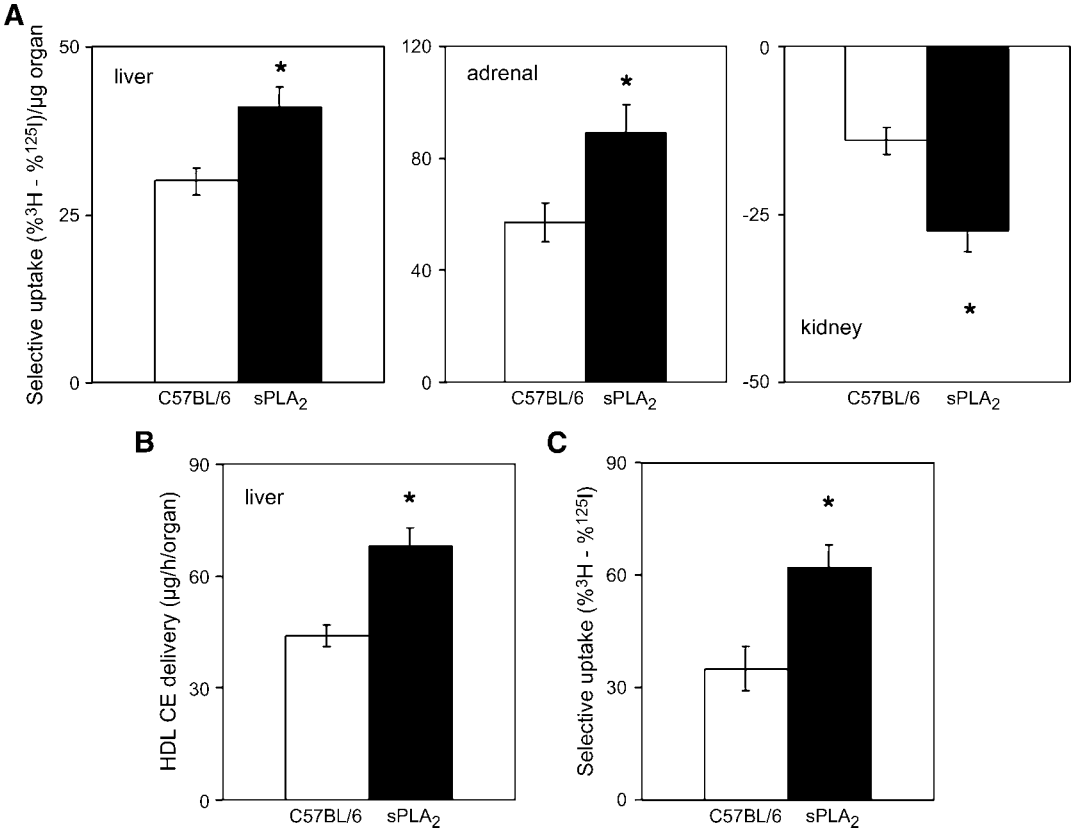

Fig. 1. Scavenger receptor class B type I (SR-BI)-mediated selective uptake of HDL cholesteryl ester is increased by secretory phospholipase A_2 (sPLA$_2$) modification of the particle in vivo and in vitro. A: In vivo selective uptake rates of HDL cholesteryl ester into liver and adrenals of sPLA$_2$ transgenic mice and C57BL/6 controls. Homologous HDL particles were isolated from the indicated mice and labeled as described in Materials and Methods. ^{125}I-tyramine-cellobiose-labeled HDL uptake (percentage of the injected dose) was subtracted from [^{3}H]cholesteryl ether-labeled HDL uptake (percentage of the injected dose) into the indicated organs to calculate selective uptake per microgram of tissue weight. Data are presented as means ± SEM. B: In vivo rate of delivery of HDL cholesteryl ester (CE) into livers of sPLA$_2$ transgenic mice and C57BL/6 controls. From the data obtained from the kinetic experiments, hepatic uptake rates per hour were calculated as described in Materials and Methods. Data are presented as means ± SEM. * Statistically significant differences between sPLA$_2$ transgenic and wild-type mice ($P < 0.05$) as assessed by the Mann-Whitney U-test; n = 6 for each group. Data are presented as means ± SEM. C: In vitro selective uptake of HDL cholesteryl esters was assessed by comparing HDL isolated from sPLA$_2$ transgenic mice and C57BL/6 controls and labeled identically as described for A. Experiments were performed as described in Materials and Methods using ldlA cells lacking LDL receptors that were stably transfected with a murine SR-BI cDNA. Data are presented as means ± SEM. * Statistically significant differences between HDL from sPLA$_2$ transgenic mice and wild-type controls ($P < 0.05$) as assessed by the Mann-Whitney U-test. Shown is one representative example (n = 3 for each condition) out of three independent experiments.

of sPLA$_2$ transgenic mice was increased significantly, as indicated by increased negative selective uptake data (−26 ± 3 vs. −14 ± 2%/µg organ; $P < 0.01$) (Fig. 1A).

In addition, we performed experiments with HDL from sPLA$_2$ transgenic mice injected into controls (see supplementary Fig. II). Consistent with the results obtained in sPLA$_2$ transgenic mice, the FCRs of sPLA$_2$-modified HDL in wild-type mice were higher than those for wild-type HDL (HDL apolipoproteins, 0.180 ± 0.019 pools/h; HDL CE, 0.214 ± 0.017 pools/h), and selective uptake into the liver (38 ± 2%/µg organ) and adrenals (80 ± 8%/µg organ) as well as HDL protein clearance by the kidneys (24 ± 3%/µg organ) were increased.

To confirm that the increased selective uptake rates of HDL CE in sPLA$_2$ transgenic mice were attributable to enzymatic modification of the HDL particle by sPLA$_2$ and were mediated by SR-BI, in vitro studies were conducted using ldlA cells stably transfected with a murine SR-BI cDNA (Fig. 1C). In these cells, selective uptake from sPLA$_2$-modified HDL was 77% higher than the selective uptake from wild-type HDL (35 ± 6 vs. 62 ± 6%/plate; $P < 0.05$) (Fig. 1C), whereas in ldlA cells not expressing SR-BI, no appreciable selective uptake was detectable.

The hepatic gene expression pattern in sPLA$_2$ transgenic mice is consistent with increased cholesterol influx and content

Compared with wild-type controls, hepatic mRNA expression of HMG-CoA reductase was decreased significantly by 66% (relative expression levels, 1.00 ± 0.06 vs. 0.34 ± 0.03; $P < 0.001$) (**Fig. 2A**) and expression of the LDL receptor was decreased significantly by 28% (relative expression levels, 1.00 ± 0.02 vs. 0.72 ± 0.04; $P < 0.01$) (Fig. 2A) in sPLA$_2$ transgenic mice, consistent with in-

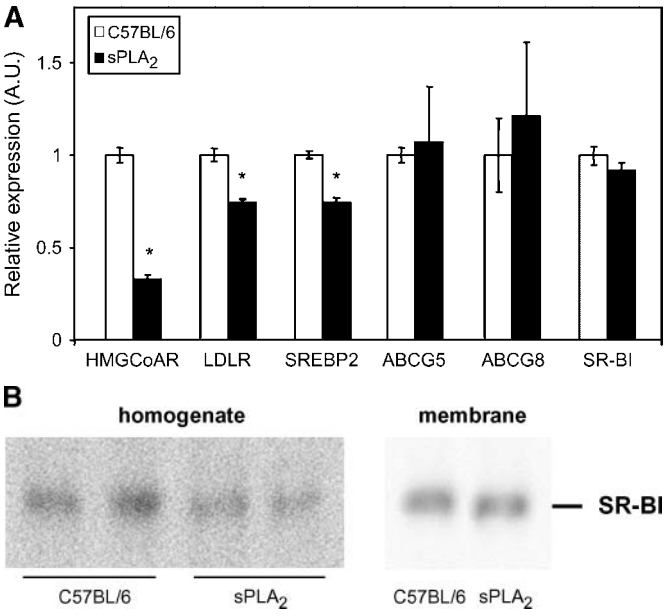

Fig. 2. Gene expression levels in livers of $sPLA_2$ transgenic mice and wild-type C57BL/6 controls. A: mRNA expression determined by quantitative real-time PCR. Measurements were performed as described in Materials and Methods. Data are presented as means ± SEM. * Statistically significant differences between $sPLA_2$ transgenic and control mice ($P < 0.05$) as assessed by the Mann-Whitney U-test; n = 6 for each group. A.U., arbitrary units; HMGCoAR, HMG-CoA reductase; LDLR, LDL receptor; SREBP2, sterol-regulatory element binding protein-2. B: Western blot analysis of total hepatic SR-BI protein expression (homogenate) and SR-BI localization to the plasma membrane (membrane) in $sPLA_2$ transgenic and control mice performed as described in Materials and Methods.

creased hepatic cholesterol uptake and content. Furthermore, hepatic expression of sterol-regulatory element binding protein-2 was decreased by 28% in $sPLA_2$ transgenic mice (1.00 ± 0.03 vs. 0.72 ± 0.03; $P < 0.001$) (Fig. 2A). In contrast, hepatic mRNA expression of ABCG5 (1.00 ± 0.06 vs. 1.07 ± 0.39; NS) (Fig. 2A) and ABCG8 (1.00 ± 0.15 vs. 1.22 ± 0.43; NS) (Fig. 2A) as well as of SR-BI (1.00 ± 0.04 vs. 0.91 ± 0.04; NS) (Fig. 2A) were unchanged in $sPLA_2$ transgenic mice compared with controls. Importantly, total hepatic SR-BI protein expression and the amount of SR-BI associated with hepatic plasma membranes also were unchanged in $sPLA_2$ transgenic mice (Fig. 2B). In addition, hepatic mRNA expression of CD36 (1.00 ± 0.06 vs. 0.93 ± 0.17; NS) and LDL receptor-related protein (1.00 ± 0.05 vs. 0.89 ± 0.05; NS) were unchanged, whereas SR-A expression was decreased (1.00 ± 0.03 vs. 0.81 ± 0.03; $P < 0.01$) in $sPLA_2$ transgenic mice.

Bile flow and biliary cholesterol excretion are unchanged in $sPLA_2$ transgenic mice

Next, we determined whether the increase in SR-BI-mediated selective uptake of HDL cholesterol into livers of $sPLA_2$ transgenic mice translates into increased biliary cholesterol secretion. Bile flow was unchanged in $sPLA_2$ transgenic mice compared with controls (6.5 ± 0.5 vs. 6.2 ± 0.4 μl/min/100 g body weight, respectively; NS) (**Fig. 3A**). However, biliary cholesterol secretion also was unchanged in $sPLA_2$ transgenic mice compared with controls (2.2 ± 0.3 vs. 1.7 ± 0.4 nmol/min/100 g body weight, respectively; NS) (Fig. 3B). In addition, no differences were detected for biliary bile salt (194 ± 20 vs. 155 ± 37 nmol/min/100 g body weight, respectively; NS) (Fig. 3B) or phospholipid secretion rates (32 ± 2 vs. 26 ± 4 nmol/min/100 g body weight, respectively; NS) (Fig. 3B) in $sPLA_2$ transgenic mice compared with wild-type controls.

Fecal neutral sterol and bile salt excretion are unchanged in $sPLA_2$ transgenic mice

Fecal bile salt excretion (2.21 ± 0.26 vs. 2.40 ± 0.30 μmol/g; NS) (**Fig. 4A**) as well as fecal neutral sterol excretion (3.58 ± 0.20 vs. 3.74 ± 0.25 μmol/g; NS) (Fig. 4B) were unchanged in $sPLA_2$ transgenic mice compared with controls. These data demonstrate that in $sPLA_2$ transgenic mice, total fecal sterol excretion is unchanged despite increased hepatic SR-BI-mediated selective uptake of HDL cholesteryl ester.

Gallstone formation is not increased in $sPLA_2$ transgenic mice

Because clinical studies reported a positive association between $sPLA_2$ expression in the gallbladder epithelium and the presence as well as the extent of cholesterol gallstone disease (27), we also assessed gallstone formation in response to feeding a lithogenic diet in our experimental model. Both $sPLA_2$ transgenic and control mice responded to feeding the lithogenic diet for 5 weeks with a significant increase (each $P < 0.001$) in bile flow that was, however, not different between the groups (15.0 ± 2.6 vs. 18.6 ± 3.5 μl/min/100 g body weight, respectively; NS). Biliary cholesterol secretion increased dramatically as well, but also without any difference between the experimental groups (54 ± 10 vs. 63 ± 9 nmol/min/100 g body weight, respectively; NS). Finally, consistent with the results obtained on biliary cholesterol secretion, the development of gallstones as assessed by polarization microscopy of gallbladder bile also was identical in both groups of mice: 58% in $sPLA_2$ transgenic mice (7 of 12; **Fig. 5**) and 67% in the controls (10 of 15; Fig. 5).

DISCUSSION

The results of this study demonstrate that in $sPLA_2$ transgenic mice, increased flux of HDL cholesterol into the liver via the SR-BI-mediated selective uptake pathway results in increased hepatic cholesterol storage and adjustment of hepatic cholesterol synthesis, but it does not translate into altered biliary cholesterol excretion in the presence of unchanged hepatic expression of SR-BI, ABCG5, and ABCG8.

In the process of RCT, hepatic SR-BI expression has been identified as a major determinant of biliary choles-

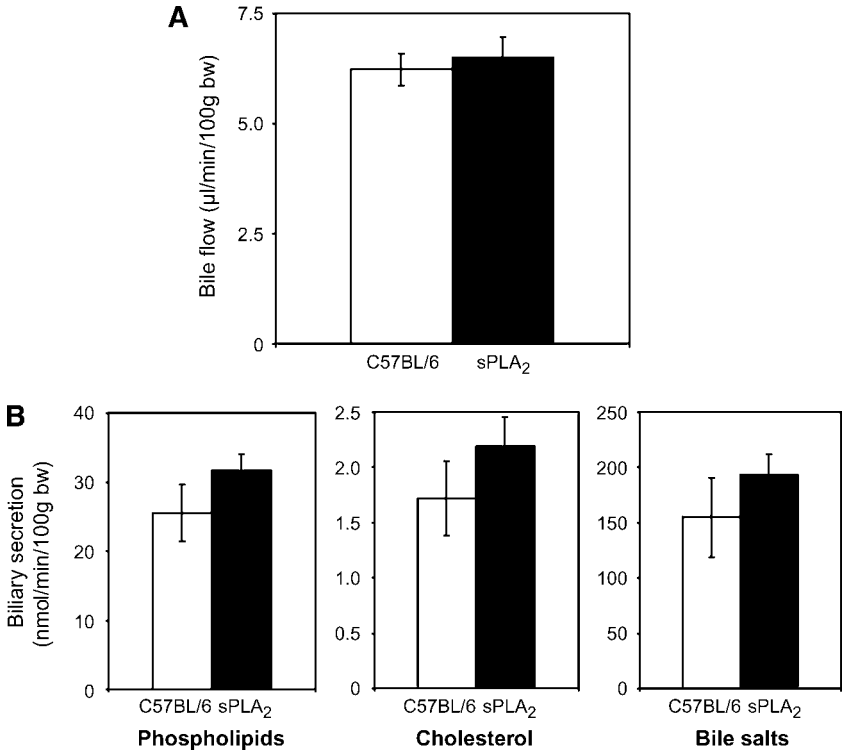

Fig. 3. Bile flow and biliary secretion rates of phospholipids, cholesterol, and bile salts in $sPLA_2$ transgenic mice and wild-type C57BL/6 controls. A: Bile flow rates determined as described in Materials and Methods. B: Biliary output rates of phospholipids, cholesterol, and bile salts. Bile was collected continuously for 40 min, substrate concentrations were determined within the bile as described in Materials and Methods, and output rates were calculated accordingly. Data are presented as means ± SEM; n = 6 for each group. BW, body weight.

terol excretion (28). Two possibilities exist for an altered HDL cholesterol uptake via SR-BI into the liver: *i*) varying hepatic SR-BI expression levels, and *ii*) lipase-mediated modification of the HDL particle. *i*) SR-BI knockout mice as well as mice with attenuated hepatic SR-BI expression have decreased levels of biliary cholesterol excretion (19, 20). Conversely, hepatic overexpression of SR-BI by transgenic or recombinant adenovirus approaches resulted in significantly increased biliary cholesterol excretion (18, 20), consistent with the concept that increasing hepatic protein levels of SR-BI promotes cholesterol elimination via the bile. *ii*) However, modification of the HDL

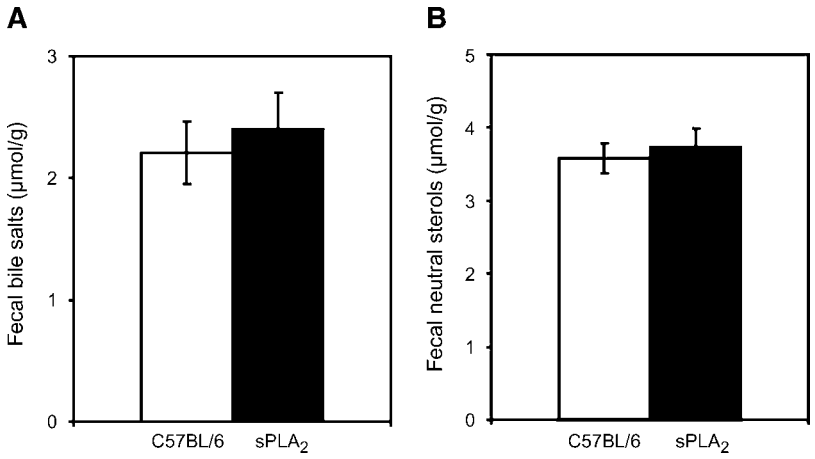

Fig. 4. Fecal excretion of neutral sterols and bile salts in $sPLA_2$ transgenic mice and wild-type C57BL/6 controls. Feces were collected from groups of two to three mice for 24 h, and concentrations of bile salts (A) and neutral sterols (B) were determined as described in Materials and Methods. Data are presented as means ± SEM; n = 8 groups each.

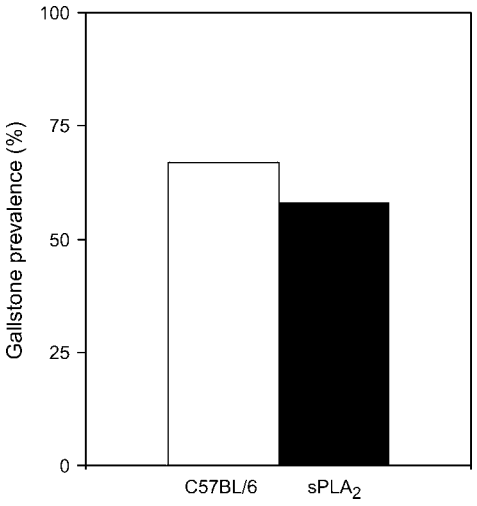

Fig. 5. Gallstone formation in $sPLA_2$ transgenic mice and wild-type C57BL/6 controls. The indicated groups of mice were fed a lithogenic diet for 5 weeks, then the formation of macroscopic gallstones was assessed as described in Materials and Methods. n = 12 for $sPLA_2$ transgenic mice and n = 15 for C57BL/6 controls.

particle by lipases such as hepatic lipase (29) or $sPLA_2$ (23, 30) renders the cholesteryl ester part of the HDL particle more susceptible to selective uptake via the SR-BI receptor pathway in the presence of unchanged SR-BI protein expression. In contrast to our findings in $sPLA_2$ transgenic mice, hepatic lipase knockout mice had unchanged hepatic cholesterol content and correspondingly also unchanged hepatic HMG-CoA reductase expression (31). The observed differences might conceivably be attributable to the different substrate specificities of the enzymes, because $sPLA_2$ acts, in contrast to hepatic lipase, exclusively as a phospholipase. However, bile flow as well as biliary secretion of cholesterol, bile salts, and phospholipids remained unchanged in hepatic lipase knockout mice, comparable to our data (31).

The unique feature of our model is that $sPLA_2$ transgenic mice have an increased flux of HDL cholesterol into the liver via SR-BI in the presence of low plasma HDL cholesterol levels and unchanged hepatic SR-BI protein expression. Our data demonstrate that increased flux via SR-BI does not per se promote an increase in biliary cholesterol excretion in the $sPLA_2$ transgenic mouse. Instead, a number of metabolic adaptations occur: *i*) increased storage of cholesterol within significantly enlarged livers; *ii*) dramatically decreased endogenous hepatic cholesterol synthesis, as indicated by HMG-CoA receptor levels of only one-third compared with controls; and *iii*) decreased LDL uptake as a consequence of decreased LDL receptor expression. The differential regulation of different hepatic metabolic and signaling pathways in $sPLA_2$ transgenic mice, namely the lack of effect on CE formation and liver X receptor activation, and on the other hand decreased expression of sterol-regulatory element binding protein-2 and its targets indicate the existence of different intracellular cholesterol pools. A further investigation of the nature of these pools will be the subject of future studies.

Our data might indicate that increased flux via SR-BI in the presence of low plasma HDL levels does not necessarily reflect increased RCT and therefore could not be considered antiatherogenic. Indeed, $sPLA_2$ transgenic mice develop increased atherosclerosis (11). On the other hand, as demonstrated by SR-BI overexpression models, increased flux of HDL cholesterol into the liver via increased hepatic SR-BI levels results in increased RCT even in the presence of low plasma HDL levels, as indicated by increased biliary cholesterol excretion and decreased atherosclerosis development (18, 20–22). Therefore, hepatic expression levels of SR-BI and not the flux via this receptor seem to be the major factor deciding the intrahepatic fate of cholesterol.

Observations in other relevant mouse models seem to support this conclusion. Apolipoprotein A-I knockout mice have significantly decreased plasma HDL cholesterol levels (32) but unchanged hepatic SR-BI expression (33). Although kinetic studies clearly established decreased HDL cholesterol delivery into the livers of apolipoprotein A-I knockout mice (32), biliary cholesterol excretion in these animals remained unaltered (20). Also, ABCA1 knockout mice have strongly decreased HDL cholesterol plasma levels, unchanged hepatic SR-BI expression, and unaltered biliary cholesterol excretion rates (25).

Recently, with the ABCG5/ABCG8 heterodimer, another key component for biliary cholesterol excretion was identified (34, 35). Both proteins have been shown to be located at the canalicular membrane in hepatocytes, suggesting that they are involved in biliary excretion. Functional studies have further demonstrated that biliary cholesterol secretion is reduced significantly in ABCG5/ABCG8 knockout mice (36). In contrast, ABCG5/ABCG8 transgenic mice exhibit a significant increase in biliary cholesterol output (37). In the $sPLA_2$ transgenic model, hepatic expression of ABCG5 and ABCG8 remained unchanged compared with that in wild-type controls, as did the biliary cholesterol secretion rates. These data indicate that in the absence of changes in the expression levels of these two important transporters, biliary cholesterol secretion is unaltered in spite of increased rates of HDL cholesterol influx into the liver.

Interestingly, $sPLA_2$ has been detected in gallbladder bile, and clinical studies reported an association between cholesterol gallstone formation and $sPLA_2$ levels in bile (27). In a follow-up study, successful treatment of patients with multiple cholesterol gallstones with ursodeoxycholic acid resulted in decreased $sPLA_2$ mRNA expression in the gallbladder epithelium and lower $sPLA_2$ protein levels in bile (38). However, our results obtained in an experimental animal model of selective $sPLA_2$ overexpression do not support these human data: *i*) no direct effects of $sPLA_2$ expression on cholesterol crystal or gallstone formation were detected when feeding transgenic mice a lithogenic diet; and *ii*) no indirect effects of $sPLA_2$ through the modulation of biliary cholesterol concentration were observed. Although $sPLA_2$ expression has profound effects on plasma cholesterol metabolism and hepatic cholesterol storage, neither biliary cholesterol levels nor secretion

were increased in $sPLA_2$ transgenic mice compared with C57BL/6 controls that lack expression of the endogenous murine $sPLA_2$ gene as a result of a frameshift mutation (5). Therefore, our data indicate that increased $sPLA_2$ expression in the gallbladder epithelium might be a consequence rather than the cause of gallstone disease. It is plausible that the expression of $sPLA_2$ as an acute phase protein increases in response to the proinflammatory environment of a gallbladder containing multiple cholesterol gallstones. However, because differences in bile acid metabolism exist between humans and mice, we cannot rule out a species-specific effect.

In summary, this study delineates the impact of $sPLA_2$ on hepatic cholesterol metabolism. Increased influx of HDL cholesterol via SR-BI into the liver by $sPLA_2$-mediated modification of the HDL particle, however, does not translate into increased biliary cholesterol elimination and therefore cannot be considered as indicative of increased RCT. These results stress the importance of increasing hepatic protein levels of SR-BI, ABCG5, and ABCG8 when considering potential pharmacological targets to increase RCT.

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