Impaired response of biliary lipid secretion to a lithogenic diet in phosphatidylcholine transfer protein-deficient mice

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Abstract Phosphatidylcholine transfer protein (PC-TP) is a cytosolic lipid transfer protein that is highly expressed in liver and catalyzes intermembrane transfer of phosphatidylcholines in vitro. To explore a role for PC-TP in the hepatocellular trafficking of biliary phosphatidylcholines, we characterized biliary lipid secretion using *Pctp*$^{-/-}$ and wild-type littermate control mice with C57BL/6J and FVB/NJ genetic backgrounds, which express PC-TP at relatively high and low levels in liver, respectively. Eight-week-old male *Pctp*$^{-/-}$ and wild-type mice were fed a chow diet or a lithogenic diet, which served to upregulate biliary lipid secretion. In chow-fed mice, the absence of PC-TP did not reduce biliary phospholipid secretion or alter the phospholipid composition of biles. However, the responses in secretion of biliary phospholipids, cholesterol, and bile salts to the lithogenic diet were impaired in *Pctp*$^{-/-}$ mice from both genetic backgrounds. Alterations in biliary lipid secretion could not be attributed to transcriptional regulation of the expression of canalicular membrane lipid transporters, but possibly to a defect in their trafficking to the canalicular membrane. These findings support a role for PC-TP in the response of biliary lipid secretion to a lithogenic diet, but not specifically in the hepatocellular transport and secretion of phosphatidylcholines.—Wu, M. K., H. Hyogo, S. K. Yadav, P. M. Novikoff, and D. E. Cohen. **Impaired response of biliary lipid secretion to a lithogenic diet in phosphatidylcholine transfer protein-deficient mice. *J. Lipid Res.* 2005.** 46: **422–431.**

Supplementary key words cholesterol • phospholipid • bile salt • bile • liver • gallstone • lipid transfer protein • bile canaliculi

During bile formation, a transhepatocellular flux of bile salts promotes the secretion of unilamellar vesicles composed of unesterified cholesterol and phospholipid molecules (1). Hepatocellular membranes, from which biliary phospholipids are derived, are composed of heterogeneous mixtures of phospholipid classes (2). By contrast, phosphatidylcholines constitute 95% of biliary phospholipids (3). The molecular mechanism(s) by which phosphatidylcholines are selected from hepatocellular membranes for biliary secretion remains poorly understood.

Phosphatidylcholine transfer protein (PC-TP) is a cytosolic lipid transfer protein and a member of the steroidogenic acute regulatory (StAR)-related transfer (START) domain-containing superfamily of proteins (4, 5). In vitro, PC-TP [recently designated StarD2 (6)] catalyzes the intermembrane transfer of phosphatidylcholines, but no other phospholipid class (7). Based on its exquisite substrate specificity (7, 8), enrichment in liver (7, 9, 10), and the observation that activity in vitro is stimulated by bile salts (11), it has been postulated that PC-TP might function to deliver phosphatidylcholines from their principal site of synthesis in the endoplasmic reticulum to the canalicular membrane for secretion into bile (1, 12, 13).

Using mice with homozygous disruption of the *Pctp* gene (*Pctp*$^{-/-}$), van Helvoort et al. (14) tested a role for PC-TP in biliary lipid secretion by mice. They observed that biliary phosphatidylcholine secretion occurred normally in *Pctp*$^{-/-}$ mice under basal conditions and when biliary secretion rates were driven to higher rates by intravenous infusion of a hydrophilic bile salt (14). Although this finding excluded PC-TP as an exclusive mechanism for hepatocellular selection and transport of biliary phosphatidylcholines, it did not eliminate the possibility that another pathway was able to compensate under these specific experimental conditions. Of the 15 mammalian START domain proteins, several [i.e., StAR (StarD1), MLN64 (StarD3), StarD4, and StarD5] appear to function in the maintenance of cholesterol homeostasis (4, 15, 16). Considering that secretion of biliary phospholipids is also upregulated in mice by dietary cholesterol supplementation

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Abbreviations: Abc, ATP binding cassette protein; PC-TP, phosphatidylcholine transfer protein; START, steroidogenic acute regulatory-related transfer; TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TMC, tauromuricholate; TUDC, tauroursodeoxycholate.

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The online version of this article (available at http://www.jlr.org) contains an additional table.

(17), we sought to determine whether *Pctp*$^{-/-}$ mice would respond normally to a dietary cholesterol challenge. Using *Pctp*$^{-/-}$ mice bred onto two distinct genetic backgrounds, we provide evidence for impaired biliary lipid secretion in *Pctp*$^{-/-}$ mice in response to a lithogenic diet containing sufficient cholesterol content to promote the formation of cholesterol gallstones. However, these data do not support a specific role for PC-TP in the hepatocellular selection of phosphatidylcholines for secretion into bile.

MATERIALS AND METHODS

Animals

Pctp$^{-/-}$ mice (14) were obtained as a generous gift from Drs. Ardy van Helvoort and Piet Borst (The Netherlands Cancer Institute, Amsterdam). These were supplied on a pure 129/Ola genetic background and on a mixed FVB/NJ and 129/Ola genetic background that was obtained by backcrossing 129/Ola *Pctp*$^{-/-}$ mice for three generations to FVB/NJ mice. In their original characterization of *Pctp*$^{-/-}$ mice, van Helvoort et al. (14) reported the unexpected finding that PC-TP expression in livers of wild-type littermate controls of mixed FVB/NJ-129/Ola genetic backgrounds was downregulated at 2 weeks of age and undetectable in 12 week old mice. To determine whether this phenomenon was strain specific, preliminary experiments examined hepatic expression of PC-TP by Western blot analysis in livers of 11-week-old wild-type mice of C57BL/6J, 129/Ola, and FVB/NJ genetic backgrounds that were purchased from the Jackson Laboratory (Bar Harbor, ME). The highest levels of PC-TP expression were observed in C57BL/6J mice, with relatively low levels in FVB/NJ mice. Adult 129/Ola mice expressed intermediate levels of PC-TP in liver. For the current experiments, we prepared *Pctp*$^{-/-}$ mice on genetic backgrounds with high (C57BL/6J) and low (FVB/NJ) expression levels. FVB/NJ and 129/Ola mixed genetic backgrounds were backcrossed three additional generations to FVB/NJ mice to achieve mice that were six generations backcrossed to FVB/NJ. *Pctp*$^{-/-}$ mice on the pure 129/Ola genetic background were backcrossed eight generations to C57BL/6J mice to obtain *Pctp*$^{-/-}$ mice on a predominantly C57BL/6J genetic background. Mice were genotyped as described by van Helvoort et al. (14).

Experimental design

Male 6–8-week-old *Pctp*$^{-/-}$ and littermate control mice were fed either a lithogenic diet consisting of 15% fat, 1.25% cholesterol, and 0.5% sodium cholate (Harlan Teklad, Madison, WI) or a chow diet consisting of 4% fat and <0.02% cholesterol (LabDiet 5001; PMI Nutrition International, Inc., Brentwood, MO) for specified periods of time. Mice were fasted for 3 h and then anesthetized with intraperitoneal injections of 87 mg/kg body weight ketamine (Fort Dodge Animal Health, Fort Dodge, IA) and 13 mg/kg body weight xylazine (Lloyd Laboratories, Shenandoah, IA). Surgery commenced at 9 AM with a midline abdominal incision. The gallbladder was first inspected for the presence of gallstones. Using a PE-10 polyethylene catheter (Becton Dickinson, Sparks, MD), the gallbladder was then cannulated immediately after distal ligation of the common bile duct (18). Bile samples were collected by diverting bile flow to an Eppendorf collection tube. Concentrated gallbladder bile (the first 10–20 μl of bile collected) was immediately analyzed by light microscopy for the presence of liquid crystals or solid cholesterol crystals. Thereafter, flow rates of hepatic bile were measured by assuming a density of 1 g/ml and weighing collection tubes every 10 min. A collection period of 1 h was required to collect sufficient volumes of bile to measure biliary lipid concentrations and compositions for each sample (see below). Samples were frozen at −80°C before analysis. Livers were then excised, rinsed with 0.15 M NaCl to remove blood, snap frozen in liquid nitrogen, and stored at −80°C. These procedures were conducted with the approval of the Institutional Animal Care and Use Committee of the Albert Einstein College of Medicine.

Analytical techniques

Biliary lipids. Biliary phospholipid concentrations were determined using an inorganic phosphorus procedure (18). Phospholipid classes were determined commercially by Lipomics Technologies, Inc. (West Sacramento, CA) (19). Briefly, equal volumes of bile samples were pooled (n = 5 mice/group) and then phospholipids were extracted into chloroform. Phospholipid classes were separated by thin-layer chromatography, after which individual phospholipid classes were scraped, extracted from silica, and quantified according to phosphorus contents. Fatty acid compositions were determined by gas chromatography. Cholesterol concentrations were measured enzymatically (Sigma, St. Louis, MO) (18). Bile salt concentrations and molecular species were determined by HPLC (18). The hydrophobic index of bile salts, a concentration-weighted average hydrophobicity of a mixture of bile salts, was determined according to the method of Heuman (20).

Hepatic PC-TP expression. Expression of PC-TP in liver was determined by Western blot analysis. Liver cytosol was prepared by homogenizing 300 mg of tissue in ice-cold buffer containing 0.25 M sucrose, 10 mM Tris, pH 7.35, 1 mM EDTA, 5 mM DTT, and Complete™ protease inhibitor (Roche, Indianapolis, IN). Samples were then centrifuged at 100,000 *g* for 1 h at 4°C. Equal amounts of cytosolic proteins (75 μg) were subjected to SDS-PAGE, transferred onto nitrocellulose membranes, and probed using a polyclonal antibody to PC-TP (21). Visualization was by enhanced chemiluminescence using goat anti-rabbit horseradish peroxidase-conjugated secondary antibody (Bio-Rad, Hercules, CA).

Expression of canalicular membrane transporters. mRNA transcripts encoding canalicular membrane transporters were quantified by Northern blot analysis. Total RNA was isolated using TRIzol reagent (Invitrogen, Carlsbad, CA). RNA (20 μg) was electrophoresed through agarose gels and transferred to nylon membranes according to standard procedures. Mouse cDNAs were kindly provided by Dr. Richard Green (Northwestern University, Chicago, IL) [ATP binding cassette protein 4 (Abcb4) and Abcb11] and Dr. David Silver (Columbia University, New York, NY) (Abcg5), and a cDNA encoding mouse β-actin was purchased from Stratagene (La Jolla, CA). cDNAs were radiolabeled with [α-^{32}P]dCTP (Perkin-Elmer Life Sciences, Torrance, CA) using a random primer kit (Invitrogen). After hybridization, blots were subjected to autoradiography and quantified by densitometry using a FluorChem 8900 Imaging system (Alpha Innotech Corp., San Leandro, CA). mRNA expression levels were normalized to β-actin expression.

Bile canalicular morphology. Morphology of bile canaliculi was assessed by both light and electron microscopy. Livers were harvested from 6–8-week-old male chow-fed mice immediately after cervical dislocation. Liver slices (2–3 mm thick) were fixed by immersion in a cold mixture of 4% paraformaldehyde and 2.5% glutaraldehyde in 0.1 M cacodylate buffer, pH 7.4, for 3 h with shaking (22). After fixation, slices were rinsed with 7.5% sucrose and stored at 4°C in 2.3 M sucrose for cryoprotection before sectioning. Livers were sectioned (10–20 μm) with a Sartorius freezing microtome (Leitz, Gottingen, Germany). To visualize

bile canaliculi, sections were incubated for ATPase activity in buffer containing 1 mM adenosine triphosphate sodium salt, 80 mM Tris maleate, pH 7.2, 10 mM $MgSO_4$ and 0.12% lead nitrate at 37°C for 45 min, followed by visualization in diluted ammonium sulfide and mounting in glycerogel (22). Mounted liver sections were examined and photographed with a Zeiss Ultraport II (Carl Zeiss, Thornwood, NY). For electron microscopy, sections were postfixed in 4% osmium tetroxide, pH 7.4, for 1 h, embedded in Epon (Polyscience, Warren, PA), and sectioned (1 μm) using an LKB ultramicrotome II (LKB, Stockholm, Sweden). Slides were examined with a Phillips EM 300 equipped with a goniometer stage (Phillips Electronic Optics, Millersville, MD).

Statistical analysis

Data are expressed as mean ± SD. Student's *t*-test assuming equal variance was used to determine statistical significance between experimental groups. Differences were considered significant for a two-tailed $P < 0.05$.

RESULTS

Figure 1 compares the expression of PC-TP in livers of 8-week-old wild-type littermate control and *Pctp*$^{-/-}$ C57BL/6J and FVB/NJ mice. Compared with wild-type FVB/NJ mice, wild-type C57BL/6J mice expressed high levels of PC-TP. Expression of PC-TP was absent in *Pctp*$^{-/-}$ mice of both genetic backgrounds.

Susceptibility to cholesterol gallstones upon challenge with a lithogenic diet is a readily detectable phenotype that differs dramatically among inbred strains of mice (23) and in part reflects the biliary response to dietary cholesterol. We examined gallbladders and gallbladder biles to determine whether cholesterol gallstone formation was accelerated in *Pctp*$^{-/-}$ mice. Mice were fed the lithogenic diet for periods ranging up to 4 weeks. Abundant liquid and solid cholesterol crystals, which precede the formation of macroscopic cholesterol gallstones (24), were detected by 2 days for FVB/NJ mice and by 9 days for C57BL/6J mice. Neither the rate of appearance of liquid and solid crystals nor the evolution of macroscopic cholesterol gallstones was influenced by the absence of PC-TP in knockout mice of either genetic background.

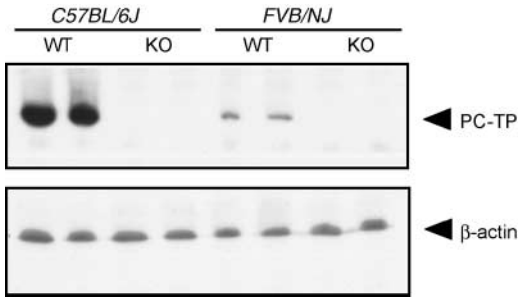

Fig. 1. Influence of genetic background on phosphatidylcholine transfer protein (PC-TP) expression in mouse liver. Western blot analysis of PC-TP in liver cytosol of 8-week-old C57BL/6J and FVB/NJ *Pctp*$^{-/-}$ [knockout (KO)] and wild-type littermate control (WT) mice.

To further characterize the influence of PC-TP on bile formation, we measured bile flow as well as biliary lipid concentrations and compositions in mice fed chow and the lithogenic diet. Because genetic background also influences biliary lipid secretion (17), we chose a common phenotypic response as a basis for choosing the duration of the dietary cholesterol challenge in C57BL/6J versus FVB/NJ mice. When fed the lithogenic diet, the formation of liquid and solid cholesterol crystals in bile is associated with mucin accumulation (24). In our experiments, occlusion of the gallbladder with mucin eventually prevented surgical cannulation. This occurred reproducibly at 7 days for C57BL/6J mice and at 18 h for FVB/NJ mice. Therefore, these time points were chosen to characterize biliary responses to the lithogenic diet in the two strains of mice in the presence or absence of PC-TP. In preliminary experiments, we also demonstrated by Western blot analysis that PC-TP expression in wild-type mice was not influenced by feeding the lithogenic diet for the specified periods of time (data not shown).

Figure 2 displays flow rates of hepatic biles. For *Pctp*$^{-/-}$ compared with wild-type C57BL/6J mice (Fig. 2A), bile flow rates were the same in chow-fed mice and in mice fed the lithogenic diet. For the same genotype, bile flow rates were higher in mice fed the lithogenic diet compared with the chow diet, but this difference only achieved statistical significance at each time point for *Pctp*$^{-/-}$ mice. In chow-

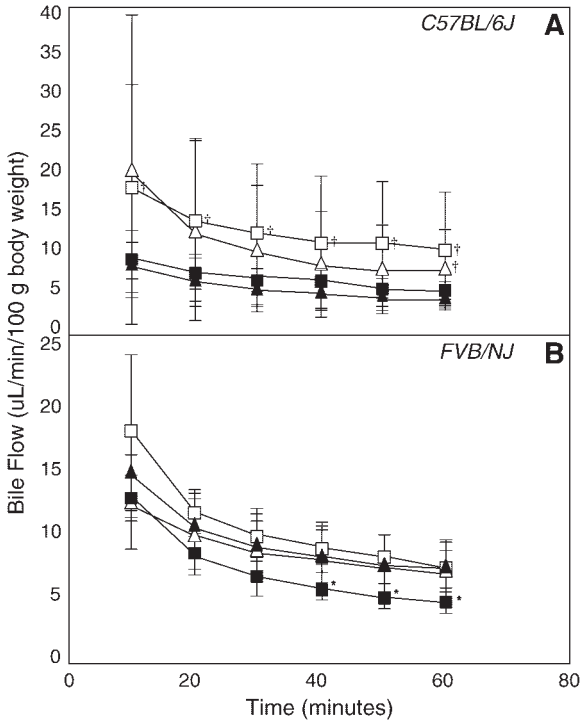

Fig. 2. Influence of PC-TP expression on bile flow. Rates of bile flow in *Pctp*$^{-/-}$ (squares) and wild-type (triangles) mice of C57BL/6J (A) and FVB/NJ (B) genetic backgrounds that were fed chow ($n \geq 4$ mice/group; closed symbols) or the lithogenic diet ($n \geq 5$ mice/group; open symbols). Error bars represent SD. * $P < 0.05$, wild-type vs. *Pctp*$^{-/-}$ mice. † $P < 0.05$, chow-fed vs. lithogenic diet-fed mice.

fed mice with the FVB/NJ genetic background (Fig. 2B), bile flow rates were the same for the first 30 min, after which flow rates in *Pctp*$^{-/-}$ mice were reduced by ~35% compared with littermate controls. The lithogenic diet did not influence bile flow in FVB/NJ mice of either genotype, nor were differences in bile flow observed between *Pctp*$^{-/-}$ and wild-type mice fed the lithogenic diet.

Figure 3 shows biliary lipid concentrations in hepatic biles. Phospholipids were readily detected in biles of chow-fed *Pctp*$^{-/-}$ C57BL/6J mice (Fig. 3A) at concentrations that were 1.3-fold higher compared with wild-type mice. The inset to Fig. 3A shows steady-state mRNA levels of Abcb4, which is rate limiting for biliary phospholipid secretion under physiological conditions (25). Expression in livers of C57BL/6J mice fed the chow diet was essentially unchanged in *Pctp*$^{-/-}$ mice. Challenge with the lithogenic diet increased biliary phospholipid concentrations in both genotypes. However, phospholipid concentrations in bile were 1.3-fold lower in the *Pctp*$^{-/-}$ mice after 7 days on the lithogenic diet. After challenge with the lithogenic diet, Abcb4 mRNA expression levels were similar in both wild-type and *Pctp*$^{-/-}$ mice (Fig. 3A, inset). In chow-fed FVB/NJ mice (Fig. 3B), phospholipid concentrations were 4-fold higher in *Pctp*$^{-/-}$ mice, whereas expression levels of Abcb4 mRNA did not differ (Fig. 3B, inset). After 18 h on the lithogenic diet, biliary phospholipid concentrations in wild-type mice increased to concentrations similar to those present in *Pctp*$^{-/-}$ mice fed chow. In *Pctp*$^{-/-}$ mice, biliary phospholipid concentrations did not change in response to the lithogenic diet. As shown in the inset to Fig. 3B, expression of Abcb4 mRNA was similar in *Pctp*$^{-/-}$ and littermate controls.

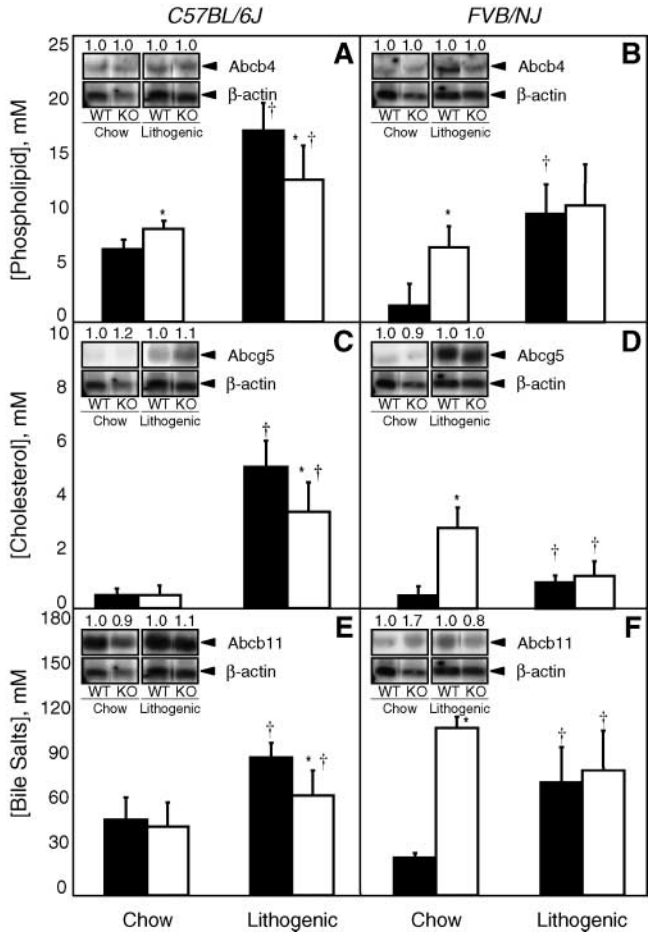

Fig. 3. Influence of PC-TP on lipid concentrations in hepatic biles. Biliary concentrations of phospholipid (A, B), cholesterol (C, D), and bile salt (E, F) were determined for wild-type (closed bars) and *Pctp*$^{-/-}$ (open bars) mice fed either a chow diet (n ≥ 4 mice/group) or the lithogenic diet (n ≥ 5 mice/group) for 7 days to C57BL/6J mice (A, C, E) or for 18 h to FVB/NJ mice (B, D, F). Insets show representative Northern blot analyses of mouse liver RNA probed for ATP binding cassette protein b4 (Abcb4; A, B), Abcg5 (C, D), and Abcb11 (E, F). Expression of each ABC transporter mRNA was normalized to β-actin. Ratios expressing fold differences in expression between *Pctp*$^{-/-}$ and littermate control mice are indicated above the blots. Error bars represent SD. * $P < 0.05$, wild-type (WT) vs. *Pctp*$^{-/-}$ [knockout (KO)] mice. † $P < 0.05$, chow-fed vs. lithogenic diet-fed mice.

In C57BL/6J wild-type and *Pctp*$^{-/-}$ mice fed the chow diet, biliary cholesterol concentrations did not differ (Fig. 3C), nor did expression levels of mRNA encoding Abcg5, one of two canalicular hemitransporters that largely control biliary cholesterol secretion (26) (Fig. 3C, inset). After challenge with the lithogenic diet, cholesterol concentrations in biles of *Pctp*$^{-/-}$ mice were 1.3-fold lower than in wild-type littermate controls. However, Abcg5 mRNA expression was the same in the livers of both genotypes. For FVB/NJ mice fed chow, biliary cholesterol concentrations were 5.9-fold higher in the absence of PC-TP (Fig. 3D). After feeding the lithogenic diet, cholesterol concentrations in biles of *Pctp*$^{-/-}$ mice decreased, whereas concentrations in wild-type mice increased. As a result, final concentrations of cholesterol were similar in both genotypes. The inset to Fig. 3D shows that livers of chow-fed FVB/NJ *Pctp*$^{-/-}$ mice expressed Abcg5 mRNA at the same level as wild-type mice and that levels were similar in both genotypes after the dietary cholesterol challenge.

Figure 3E demonstrates that biliary bile salt concentrations in chow-fed C57BL/6J mice were not affected by the absence of PC-TP. The same was true for the mRNA expression levels of Abcb11 (Fig. 3E, inset), the canalicular bile salt export pump that rate limits biliary bile salt secretion (27, 28). Bile salt concentrations increased in both *Pctp*$^{-/-}$ and wild-type mice after challenge with the lithogenic diet. Although bile salt concentrations were 1.3-fold lower in knockout mice, hepatic expression levels of Abcb11 mRNA were the same in *Pctp*$^{-/-}$ and wild-type mice (Fig. 3E, inset). In FVB/NJ mice fed chow, bile salt concentrations were markedly increased (4.3-fold) in *Pctp*$^{-/-}$ compared with wild-type mice (Fig. 3F). These mice also displayed 1.7-fold higher expression of Abcb11 mRNA than did wild-type mice. After feeding the lithogenic diet, bile salt concentrations decreased in *Pctp*$^{-/-}$ mice but increased in wild-type controls to concentrations similar to those observed in knockout mice. Abcb11 mRNA levels in livers of wild-type and *Pctp*$^{-/-}$ FVB/NJ mice were similar after challenge with the lithogenic diet (Fig. 3F, inset).

To assess whether PC-TP expression influenced classes or fatty acid compositions of biliary phospholipids, we an-

alyzed biles from C57BL/6J *Pctp*$^{-/-}$ mice and their littermate controls, which express abundant PC-TP in liver (Fig. 1). As shown in **Fig. 4A**, biliary phospholipids in both *Pctp*$^{-/-}$ and wild-type mice were composed principally of phosphatidylcholines, with minor contributions from phosphatidylethanolamines and sphingomyelins. Challenge with the lithogenic diet did not substantially alter the distribution of phospholipid classes (Fig. 4B). Compositions of fatty acyl chains that constituted the individual phospholipid classes are presented in the supplementary table online. For each phospholipid class, 16:0 and 18:2n6 accounted for the majority of fatty acid species. There were no appreciable differences between *Pctp*$^{-/-}$ and wild-type mice fed either chow or the lithogenic diet.

To determine the influence of PC-TP expression on biliary bile salt compositions, we analyzed the molecular species of bile salts in hepatic bile. In *Pctp*$^{-/-}$ C57BL/6J mice fed chow (**Fig. 5A**), the relative concentration (mole percent) of tauromuricholate (TMC) was reduced, whereas tauroursodeoxycholate (TUDC) and taurocholate (TC) were each increased compared with wild-type mice. The relative concentrations of taurochenodeoxycholate (TCDC) and taurodeoxycholate (TDC) were similar in both genotypes. After feeding the lithogenic diet (Fig. 5C), TMC decreased to similar relative concentrations in *Pctp*$^{-/-}$ and wild-type mice. TUDC increased in both genotypes but remained higher in the *Pctp*$^{-/-}$ mice. Relative concentrations of TC increased in wild-type mice but remained unchanged in *Pctp*$^{-/-}$ mice. Relative concentrations of TCDC and TDC increased to similar levels. In chow-fed FVB/NJ mice (Fig. 5B), the relative concentration of TC was higher in *Pctp*$^{-/-}$ mice than in wild-type mice, whereas other bile salts were lower. In response to the lithogenic diet (Fig. 5D), the percentage of TUDC was higher in *Pctp*$^{-/-}$ mice. All other bile salt species were similar. **Figure 6** shows bile salt hydrophobic indices in *Pctp*$^{-/-}$ and wild-type mice. There were no differences in the hydrophobic indices of bile salts between *Pctp*$^{-/-}$ and wild-type mice on either genetic background when fed chow. Although the lithogenic diet increased the hydrophobic index in mice of both genetic backgrounds, there was no effect of PC-TP expression in either mouse strain.

In addition to bile salt secretion rates, the coupling of bile salt to phospholipid and cholesterol during biliary lipid secretion is controlled by both the hydrophobicity of biliary bile salts (29, 30) and the composition of biliary phospholipids (31). Because PC-TP expression minimally affected bile salt hydrophobicity, phospholipid composition, or bile flow, this allowed us to use the data in Fig. 3 to determine whether the differences in biliary phospholipid and cholesterol secretion were directly attributable to changes in bile salt secretion. **Table 1** gives the coupling ratios among the biliary lipids for *Pctp*$^{-/-}$ and wild-type mice of both genotypes for each diet used. The coupling ratios denote the moles of phospholipid or cholesterol secreted per mole of secreted bile salt (i.e., phospholipid/bile salt and cholesterol/bile salt, respectively) or the moles of cholesterol secreted per mole of secreted phospholipid (cholesterol/phospholipid). Consistent with its

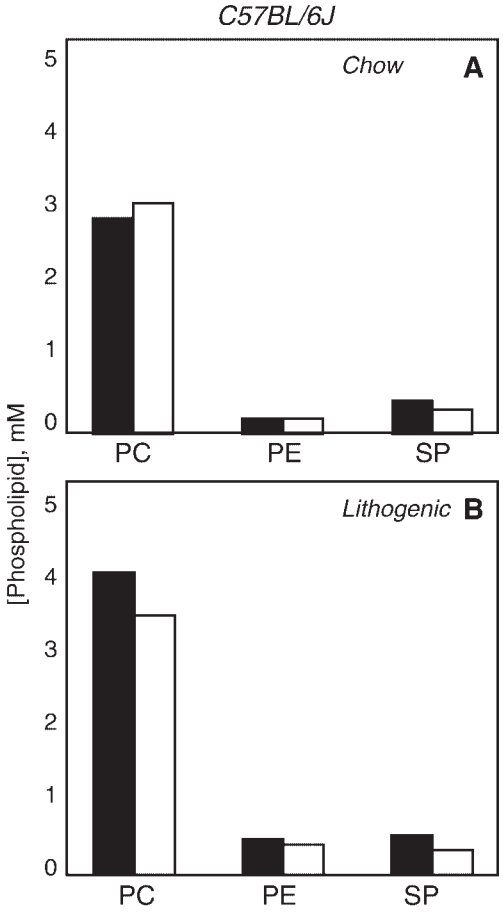

Fig. 4. Influence of PC-TP on biliary phospholipid classes. Biliary concentrations of phosphatidylcholine (PC), phosphatidylethanolamine (PE), and sphingomyelin (SP) in bile were measured for pooled bile samples (n = 5 mice/group) obtained from C57BL/6J wild-type (closed bars) and *Pctp*$^{-/-}$ (open bars) mice fed either chow (A) or the lithogenic diet (B).

effects on bile salt hydrophobicity and secretion rates (30, 32), the lithogenic diet resulted in significant changes in all coupling ratios except the phospholipid/bile salt ratio in C57BL/6J mice. However, with the exception of a modest decrease in the cholesterol/phospholipid ratio in chow-fed *Pctp*$^{-/-}$ C57BL/6J mice, coupling ratios were not affected by genotype for either genetic background. This indicates that changes in phospholipid and cholesterol secretion into bile were attributable to changes in bile salt secretion.

Although the marked increase in bile salt secretion in *Pctp*$^{-/-}$ FVB/NJ mice was accompanied by a modest increase in Abcb11 mRNA expression (Fig. 3F), transcriptional regulation of canalicular transporters did not explain the differences in biliary lipid secretion that occurred in the absence of PC-TP expression, particularly in response to the lithogenic diet. Vesicular trafficking of these transporters to the canalicular membrane represents another mechanism for controlling biliary lipid secretion (33–35). To garner evidence to suggest that vesicle trafficking to the canalicular membrane might be impaired in the absence of PC-TP expression, we characterized

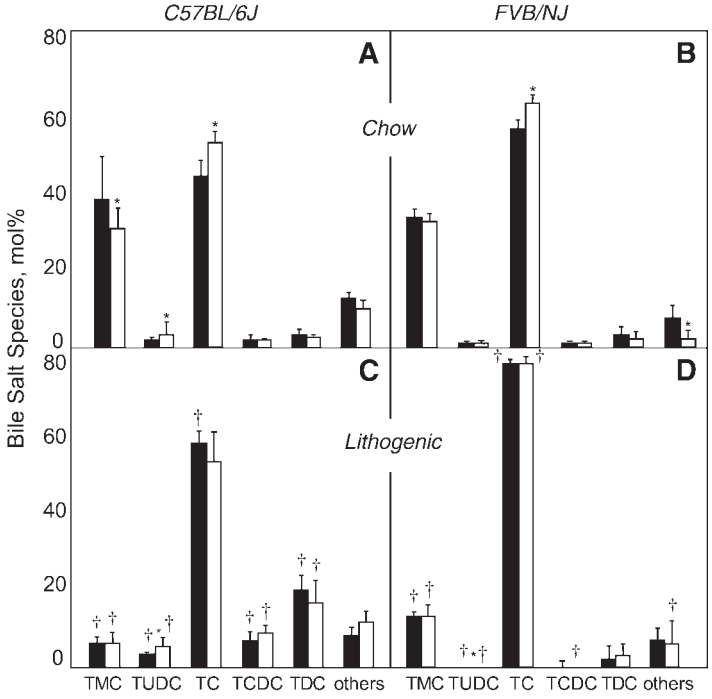

Fig. 5. Influence of PC-TP on bile salt molecular species. Bile salt molecular species were determined for wild-type (closed bars) and *Pctp*$^{-/-}$ (open bars) mice fed either chow (n ⩾ 4 mice/group; A, B) or the lithogenic diet (n ⩾ 5 mice/group; C, D) to C57BL/6J mice for 7 days (A, C) or FVB/NJ mice for 18 h (B, D). TC, taurocholate; TCDC, taurochenodeoxycholate; TDC, taurodeoxycholate; TMC, tauromuricholate; TUDC, tauroursodeoxycholate. Error bars represent SD. * $P < 0.05$, wild-type vs. *Pctp*$^{-/-}$ mice. † $P < 0.05$, chow-fed vs. lithogenic diet-fed mice.

the morphology of bile canaliculi in *Pctp*$^{-/-}$ and wild-type mice fed chow (**Fig. 7**). As previously described, ATPase staining specifically labels bile canaliculi (22). In livers of wild-type FVB/NJ mice, there was a normal pattern of interconnecting bile canaliculi (Fig. 7A). By contrast, in the absence of PC-TP expression, bile canaliculi in *Pctp*$^{-/-}$ FVB/NJ mice demonstrated extensive branching at the membrane surface (Fig. 7C). Not shown are micrographs for C57BL/6J mice, which demonstrated the same findings. Electron microscopy was performed to gain further insight into the morphological changes in bile canaliculi. Figure 7B shows a representative electron micrograph that corresponds to the image in Fig. 7A. In wild-type mice, there was a normal appearance of bile canaliculi, as well as the appropriate size and placement of junctional complexes. Fig. 7D shows a representative electron micrograph corresponding to Fig. 7C. In *Pctp*$^{-/-}$ mice, bile canaliculi appeared more tortuous, as did the junctional complexes. In addition, there was an accumulation of vesicles in the subapical region near the bile canaliculi.

DISCUSSION

The hepatocellular mechanism(s) responsible for the selection, transport, and secretion of biliary phosphatidylcholines is incompletely understood. Evidence has been presented that Abcb4 (Mdr2), which rate limits biliary phosphatidylcholine secretion at the canalicular membrane, may function as a specific flippase for phosphatidylcholines, thereby providing a selective mechanism for phosphatidylcholines (36). Because of the high rates of

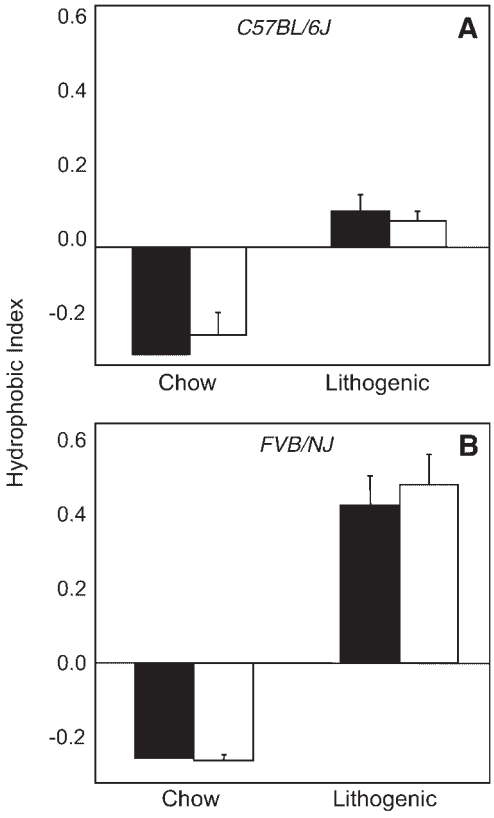

Fig. 6. The absence of PC-TP does not affect bile salt hydrophobicity. Hydrophobic indices of biliary bile salts for wild-type (closed bars) and *Pctp*$^{-/-}$ (open bars) mice after feeding either chow (n ⩾ 4 mice/group) or the lithogenic diet (n ⩾ 5 mice/group) to C57BL/6J mice for 7 days (A) or FVB/NJ mice for 18 h (B). Error bars represent SD.

TABLE 1. Coupling ratios of biliary lipids

Genetic Background	Genotype	Phospholipid/Bile Salt		Cholesterol/Bile Salt		Cholesterol/Phospholipid	
		Chow Diet	Lithogenic Diet	Chow Diet	Lithogenic Diet	Chow Diet	Lithogenic Diet
C57BL6/J	Wild type	0.153 ± 0.058	0.209 ± 0.050	0.014 ± 0.003	0.072 ± 0.018[a]	0.099 ± 0.024	0.348 ± 0.059[a]
	Pctp$^{-/-}$	0.201 ± 0.061	0.217 ± 0.037	0.016 ± 0.005	0.079 ± 0.056[a]	0.082 ± 0.017[b]	0.419 ± 0.303[a]
FVB/NJ	Wild type	0.070 ± 0.013	0.141 ± 0.022[a]	0.021 ± 0.009	0.012 ± 0.004[a]	0.307 ± 0.193	0.086 ± 0.035[a]
	Pctp$^{-/-}$	0.064 ± 0.006	0.128 ± 0.047[a]	0.028 ± 0.008	0.014 ± 0.003[a]	0.436 ± 0.101	0.128 ± 0.047[a]

[a] $P < 0.05$, chow diet (n ≥ 4 mice/group) vs. lithogenic diet (n ≥ 5 mice/group).
[b] $P < 0.05$, wild type vs. *Pctp*$^{-/-}$.

Abcb4-mediated biliary phosphatidylcholine secretion (37, 38), hepatocytes would be expected to possess efficient mechanisms to replenish the canalicular membrane as phosphatidylcholine molecules are removed during bile formation. Earlier observations that microtubule-disrupting agents could inhibit biliary secretion of cholesterol and phospholipid (39–41) suggested that transport of vesicles along microtubules could provide a resupply route (reviewed in 37). Although the existence of a vesicular pathway has been supported by observations using confocal laser scanning microscopy to track the movement of fluorescent phospholipid and cholesterol analogs to the canalicular membrane of HepG2 cells in culture (42, 43), neither the mechanisms nor the quantitative contribution of a vesicular pathway is understood. Studies demonstrating localization of the liver-specific enzyme, phosphatidylethanolamine *N*-methyltransferase, to the canalicular membrane (44, 45) suggest that some biliary phosphatidylcholines may be synthesized at their site of secretion into bile. In addition, recent observations in polarized HepG2 cells indicate that rapid nonvesicular transport, perhaps via PC-TP, contributes significantly to the delivery of phosphatidylcholines to the canalicular membrane (42).

The goal of this study was to further examine a role for PC-TP in biliary lipid secretion, particularly in response to a dietary cholesterol challenge. In an initial study of *Pctp*$^{-/-}$ mice, van Helvoort et al. (14) reported no differences in biliary lipid secretion between knockout and wild-type mice. They also made the unexpected observation that PC-TP expression in livers of the wild-type FVB/NJ-129/Ola littermate control mice was downregulated at 2 weeks of age and essentially undetectable at 12 weeks. Their experiments clearly showed that biliary phosphatidylcholine secretion occurs in *Pctp*$^{-/-}$ mice. However, because of the absence of expression in wild-type mice, it remained unclear whether PC-TP plays a role in hepatocellular trafficking of biliary phospholipids. To address this issue, we used *Pctp*$^{-/-}$ mice that were bred onto two distinct genetic backgrounds in which PC-TP expression in adult wild-type mice was present at relatively high (C56BL/6J) and low (FVB/NJ) levels in liver.

In our experiments, we stimulated biliary phospholipid secretion in mice using a high-fat/high-cholesterol, cholate-containing lithogenic diet (23). When challenged with this diet, intestinally derived cholesterol becomes a major source of biliary cholesterol (46) and the bile salt pool of mice becomes enriched with much more hydro-

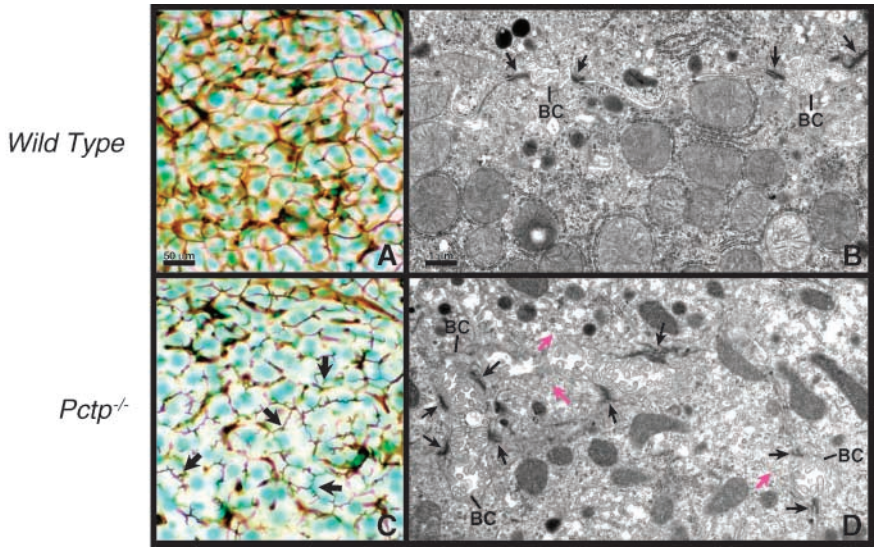

Fig. 7. Abnormal morphology of bile canaliculi in *Pctp*$^{-/-}$ mice. Bile canaliculi were stained for ATPase activity using liver sections from chow-fed FVB/NJ wild-type (A) and *Pctp*$^{-/-}$ (C) mice. Branching of bile canaliculi is evident only in *Pctp*$^{-/-}$ mice (arrows in C). Electron micrographs of livers from FVB/NJ wild-type (B) and *Pctp*$^{-/-}$ (D) mice. Bile canaliculi are denoted by BC. Junctional complexes are shown by black arrows. Subapical vesicles are indicated by pink arrows.

phobic bile salts than are typically present (17). As a result, secretion rates of bile salts, phospholipids, and cholesterol are all increased dramatically (17). Because of disproportionate increases in cholesterol secretion, biles also become supersaturated with cholesterol (24). When fed the lithogenic diet for sufficient periods of time, genetically susceptible mouse strains, including C57BL/6J (23) and FVB/NJ (47), form cholesterol gallstones. In the current study, we first tested whether cholelithiasis might be accelerated in *Pctp*$^{-/-}$ mice as a result of relative hyposecretion of biliary phosphatidylcholines. However, we did not observe an effect of PC-TP expression on the appearance of cholesterol gallstones or their precursors (48) in mice of either genetic background.

Given that the pathogenesis of cholesterol gallstone formation is multifactorial (49), the absence of a gallstone-related phenotype in these experiments did not a priori exclude a role for PC-TP in biliary phospholipid secretion. We therefore characterized biliary lipid secretion at baseline and in response to the lithogenic diet. We observed that phospholipids were secreted into biles of chow-fed *Pctp*$^{-/-}$ mice of both C57BL/6J and FVB/NJ genetic backgrounds. Moreover, the absence of PC-TP did not appreciably influence the relative abundance of biliary phosphatidylcholines in the C57BL/6J strain, in which PC-TP is normally expressed at high levels. Taken together, these findings argue strongly against a physiological role for PC-TP in biliary phosphatidylcholine secretion under basal conditions.

In *Pctp*$^{-/-}$ FVB/NJ mice fed chow, there was marked upregulation in the biliary concentrations of bile salts, phospholipids, and cholesterol. Bile salts promote the biliary secretion of phospholipids and cholesterol in proportion to their rate of secretion (32) and hydrophobicity (30). Whereas bile salt hydrophobicity was not influenced by PC-TP expression, the marked increase in bile salt secretion rates may have been attributable, in part, to transcriptional upregulation of Abcb11. Because transcriptional upregulation of Abcb11 occurs primarily when hydrophobic bile salts activate the farnesoid X receptor (50), our current experiments do not explain the mechanism by which the absence of PC-TP upregulates Abcb11. Nevertheless, the coupling ratios of biliary lipids reveal that increases in biliary phospholipid and cholesterol secretion in *Pctp*$^{-/-}$ FVB/NJ mice were entirely attributable to increased bile salt secretion.

A significant phenotype of *Pctp*$^{-/-}$ mice was revealed by considering the changes in biliary lipid concentrations in response to the lithogenic diet (Fig. 3). In C57BL/6J *Pctp*$^{-/-}$ mice fed the lithogenic diet, biliary concentrations of phospholipids, cholesterol, and bile salts were reduced compared with those in wild-type mice. Because concentrations of each lipid were similar in chow-fed wild-type and *Pctp*$^{-/-}$ animals, the responses (i.e., fold increases) to the lithogenic diet were all reduced in the knockout mice (wild type: bile salt, 1.8; phospholipid, 2.7; cholesterol, 9.7; *Pctp*$^{-/-}$: bile salt, 1.5; phospholipid, 1.7; cholesterol, 7.0). For FVB/NJ wild-type and *Pctp*$^{-/-}$ mice, biliary lipid concentrations were similar after feeding the lithogenic diet. However, lipid concentrations in chow-fed wild-type mice were lower than those in their *Pctp*$^{-/-}$ counterparts. Therefore, the responses in biliary lipid secretion rates were also reduced in *Pctp*$^{-/-}$ mice on the FVB/NJ genetic background (wild type: bile salt, 2.9; phospholipid, 6.0; cholesterol, 1.9; *Pctp*$^{-/-}$: bile salt, 0.8; phospholipid, 1.5; cholesterol, 0.4). By contrast, responses of mRNA expression of canalicular lipid transporters were largely unaffected in the absence of PC-TP expression.

Activities of the canalicular transporters are rate limiting for biliary lipid secretion (25–27, 51), and emerging evidence has demonstrated that hepatocellular trafficking of vesicles containing Abcb4 (33, 34), Abcg5/Abcg8 (35), and Abcb11 (34) plays a key role in the posttranslational regulation of biliary lipid secretion. Moreover, Müller et al. (52) have demonstrated that lithogenic diet-induced increases in biliary lipid secretion rates in gallstone-susceptible mice may be mediated by translocation of transporter proteins to the canalicular membrane. Consistent with the possibility that trafficking of ABC transporters to the canalicular membrane might be impaired in the absence of PC-TP are our observations that bile canaliculi in *Pctp*$^{-/-}$ mice were tortuous and that there was a subapical collection of vesicles. Treatment with colchicine, an agent that disrupts microtubules, can lead to a similar appearance of the canalicular membrane by electron microscopy (53). However, in contrast to what we observed in *Pctp*$^{-/-}$ mice, this agent selectively impairs biliary secretion of phospholipid and cholesterol without inhibiting biliary bile salt secretion (39). Additional systematic studies will be required to determine whether and by what mechanism the absence of PC-TP leads to impaired canalicular localization of lipid transporters.

In summary, our finding are in agreement with those of van Helvoort and colleagues (14) demonstrating that PC-TP is not required for biliary phospholipid secretion and does not appear to act as a shuttle protein that specifically delivers phosphatidylcholines to the canalicular membrane for secretion into bile. However, the current study reveals an impaired response of biliary lipid secretion to a lithogenic diet as a phenotype of the *Pctp*$^{-/-}$ mouse.

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