Ablating L-FABP in SCP-2/SCP-x null mice impairs bile acid metabolism and biliary HDL-cholesterol secretion

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Bile acid synthesis, intracellular transport, and excretion represent the major route of cholesterol catabolism and elimination in mammals. Canaliculal bile acid secretion is the rate-limiting step in bile formation and drives biliary cholesterol secretion (60, 64, 80). Bile acids have also been shown to be important metabolic regulators of glucose, lipid, and energy homeostasis (43). Consequently, hepatic primary bile acid synthesis is a tightly regulated, albeit complex, pathway of chemical modifications to cholesterol involving up to 17 different enzymes localized in different subcellular compartments, including the endoplasmic reticulum, mitochondrion, peroxisome, and cytoplasm (64). The mechanism of transport of the relatively hydrophobic intermediates in hepatic bile acid synthesis from one compartment to another and, finally, to the bile canaliculus for secretion in mammals is largely unknown and represents a challenging issue in current bile acid research (1, 24, 42, 50, 64, 80).

In nonmammalian vertebrates (e.g., fish, amphibians, reptiles, and birds), a liver bile acid-binding protein (L-BABP) is thought to be involved in intracellular hepatic bile acid transport (50). L-BABP, also referred to as liver “basic” fatty acid-binding protein because of its basic isoelectric point (pI ~9.0) (50), has been sequenced, isolated, and purified, and its protein structure has been determined by X-ray diffraction studies (66). L-BABP is a member of the fatty acid-binding protein (FABP) family, all members of which share in common a three-dimensional structure comprising a 10-stranded antiparallel β-barrel with two short α-helices situated between the first and second β-strands (74). L-BABP binds two molecules of bile acids (50, 56). In contrast to these findings in nonmammalian vertebrate species, a L-BABP directly involved in intracellular bile acid transport has not been identified in any mammalian species (24, 50).

While mammalian liver does not contain L-BABP, at least three other classes of proteins have been shown to bind bile acids in vitro: liver FABP (L-FABP), glutathione S-transferase (GST), and 3α-hydroxysteroid dehydrogenase (3α-HSD) (73, 78). While the functional significance of GST and 3α-HSD in intracellular bile acid transport is unknown, increasing data suggest that L-FABP might function in place of L-BABP, which is absent from mammalian liver. Although L-FABP binds bile acids only at a single site and with lower affinity than L-BABP (13–16, 25, 35, 79), L-FABP is the most prevalent hepatic FABP family member and BABP in murine (2–6% of cytosolic protein; 200–400 µM) and human (7–10% of cytosolic protein; 700–1,000 µM) liver (16, 48). Furthermore, L-FABP also binds cholesterol, the substrate for bile acid synthesis (17, 44). Several functional studies in rodents further support a role for L-FABP in bile acid metabolism. For example, rat L-FABP inhibits liver microsomal sulfation of glycolithocholic acid in vitro (70). While cross-linking studies with a photoaffinity-labeled bile acid also suggest that L-FABP is essential for bile acid uptake and intracellular transport in rat liver hepatocytes, this does not distinguish effects of cross-linking to membrane bile acid transporters vs. L-FABP (15). However, an early study with L-FABP null mice provided the first physiological evidence suggesting a role for L-FABP in the metabolism of cholesterol and bile salts (45).

While not a BABP per se, the sterol carrier protein (SCP)-2/SCP-x gene’s protein translation products also appear to be involved in bile acid synthesis and biliary secretion of bile acids. By binding and transferring cholesterol (12, 23, 44, 54,
SCP-2 stimulates liver microsomal cholesterol 7α-hydroxylase (the rate-limiting enzyme in hepatic bile acid synthesis) in vitro (37). SCP-2 overexpression increases bile acid synthesis and biliary secretion in mice and in isolated rat and human hepatocytes (2, 59). While SCP-x, the alternate transcriptional product of the SCP-2/SCP-x gene, also transfers cholesterol, perhaps more importantly it is the only known peroxisomal branched-chain ketothiolase that oxidizes the cholesterol side chain required for bile acid synthesis (68). Although ablation of the SCP-2/SCP-x gene decreases bile acid synthesis and biliary bile acid secretion in mice, interpretation of this finding is complicated by concomitant severalfold upregulation of L-FABP (22, 36, 69, 75).

In the present study, our laboratory investigated bile acid metabolism and biliary bile acid and high-density lipoprotein (HDL)-cholesterol secretion in male mice lacking L-FABP (LKO), SCP-2/SCP-x [double knockout (DKO)], or L-FABP/SCP-2/SCP-x [triple knockout (TKO)]. Loss of L-FABP protein/ function significantly impacted bile acid levels in liver (LKO and TKO) and bile (LKO) and also altered biliary bile acid composition and hydrophobicity. Loss of L-FABP (LKO and TKO) decreased hepatic HDL-derived 22-(N-(7-nitrobenz-2-oxa-1,3-diazol-4-yl)amino)-23,24-bisnor-5-cholen-3β-ol (NBD-cholesterol) uptake and biliary secretion. In contrast, while loss of SCP-2/SCP-x alone did not alter hepatic or biliary bile acid concentration, it did alter biliary bile acid composition, cholesterol saturation index, and hydrophobicity. However, SCP-2/SCP-x ablation had no impact on hepatic uptake and biliary secretion of HDL-derived NBD-cholesterol. These results demonstrate that L-FABP and SCP-2/SCP-x have important, but distinct, roles in cholesterol/bile acid homeostasis in mammals.

**EXPERIMENTAL PROCEDURES**

**Materials.** Protein Assay Kit I (catalog no. 500-0001, bovine γ-globulin) was purchased from Bio-Rad (Hercules, CA). The following diagnostic kits were obtained from Wako Chemicals (Richmond, VA): cholesterol E (total cholesterol), free cholesterol E (free cholesterol), and phospholipid C (PL). The total bile acid diagnostic kit was purchased from Diazyme Labs (Poway, CA). NBD-cholesterol was obtained from Molecular Probes/Invitrogen (Eugene, OR). [1,2,6,7-3H(N)]cholesterol (22.5 Ci/mmol) was purchased from NEN Life Science/Perkin Elmer (Waltham, MA). Bile acid standards [choleic acid (CA), α-muricholic acid (MCA), β-MCA, tauro-β-MCA (T-MCA), tauroliothocholic acid (T-LCA), tauroursododeoxycholic acid, tauro-CA (T-CA), taurochenodeoxycholic acid, and taurodeoxycholic acid] were obtained from Steraloids (Newport, RI). Purified human HDL was purchased from EMD Biosciences (San Diego, CA). TaqMan One-Step PCR Master Mix reagent kit and gene-specific assays for ATP-binding cassette transporters G5 and G8 [ABC5 (Mm01226965_m1) and ABCG8 (Mm0045977_m1)], cholesterol 7α-hydroxylase [Cyp7a1 (Mm0048152_m1)], sodium taurocholate-cotransporting polypeptide [NTCP (Mm01302718_m1)], and organic anion-transporting polypeptides 1 and 2 [OAT1P1 (Mm01267414_m1) and OATP2 (Mm00460672_m1)] were obtained from Applied Biosystems (Foster City, CA). Rabbit or goat polyclonal antibody to mouse β-actin (sc-7788), bile salt export pump [BSEP (sc-179294)], steroid 27-hydroxylase [Cyp27a1 (sc-14835)], farneosid x receptor [FXR (sc-13063)], liver x receptor-α [LXRα (sc-1201)], multidrug-resistance-associated protein 2 [MRP2 (sc-5770)], Niemann-Pick C1-like 1 (sc-49061), short heterodimer partner [SHP (sc-15283)], and scavenger receptor class B type 1 [SR-B1 (sc-32342)] were purchased from Santa Cruz Biotechnology (Dallas, TX). Rabbit polyclonal antibody to mouse acetyl-CoA acetyltransferase-2 [ACAT2 (ab66259)], cytochrome c oxidase subunit 4 (COX4-4 [ab16056]), and multidrug-resistance-3 P-glycoprotein [Mdr3 (ab71792)] were obtained from Abcam (Cambridge, MA). Rabbit polyclonal antibody to peroxisome proliferator-activated receptor (PPAR)-α [PPARα (PA1-822A)] was purchased from Pierce Antibody (Rockford, IL). Mouse monoclonal antibody to mouse GAPDH (MAB 374) was obtained from Millipore (Billerica, MA). Rabbit polyclonal antibody to 3α-HSD was purchased from US Biological (Peabody, MA). Alkaline phosphatase-conjugated goat polyclonal antibody to rabbit IgG (product no. A3687) and rabbit polyclonal antibody to goat IgG (product no. A4187) were obtained from Sigma-Aldrich (St. Louis, MO). Alkaline phosphatase-conjugated rabbit polyclonal antibody to mouse IgG (product no. ab6729-1) was purchased from Abcam. All reagents and solvents were of the highest grade available.

**Animals.** All animal protocols were approved by the Institutional Animal Care and Use Committee at Texas A & M University. Male (6 wk old, 20–30 g body wt) inbred C57BL/6Ncr wild-type (WT) mice were obtained from the National Cancer Institute (Frederick Cancer Research and Development Center, Frederick, MD). L-FABP gene-ablated/null (LKO) mice (46), SCP-2/SCP-x gene-ablated/null (DKO) mice (5), and L-FABP/SCP-2/SCP-x gene-ablated/null (TKO) mice (75) were generated in our laboratory as previously described. Each gene-ablated mouse strain was backcrossed to the C57BL/6Ncr background to >10 generations. All mice were kept in a temperature-controlled (25°C) facility under a 12:12-h light-dark cycle. The animals had access to food (phytol/phytostrogen-free, 5 g fat/100 g diet; no. D11243, Research Diets, New Brunswick, NJ) and water ad libitum. All mice were monitored quarterly for known rodent pathogens and were determined to be pathogen-free.

**Animal euthanasia and tissue collection.** Prior to euthanasia, each animal was fasted for 12 h, weighed, and anesthetized with tribromoethanol (Avertin). Blood was collected from the mouse via cardiac puncture into a polypropylene microtube. The collected blood was immediately processed to serum, serum volume was measured, and the serum was flash-frozen and stored at −80°C. The mouse was euthanized by cervical dislocation. The liver was removed, carefully blotted dry, weighed, flash-frozen with 0.5 ml of phosphate-buffered saline (pH 7.4) in dry ice, and stored at −80°C. A small (0.1–0.2 g) piece of each freshly isolated liver was flash-frozen in RNA stabilizing buffer (RNAzol, Tel-Test, Austin, TX) and stored at −80°C; this portion of liver was used for quantitative RT-PCR (see Quantitative real-time RT-PCR). Bile was collected from the gallbladder, bile volume was measured, and the bile was flash-frozen in dry ice and stored at −80°C.

**Lipid analysis.** A small (~0.1 g) portion of mouse liver was homogenized in 0.5 ml of phosphate-buffered saline (pH 7.4) as follows. The weighed liver portion was minced extensively and homogenized for 5 min on ice in a 1.5-ml microcentrifuge tube with use of a motor-driven pestle (Tekmar, Cincinnati, OH) operating at 2,000 rpm. Additional homogenization was accomplished by sonication on ice utilizing a sonic dismembrator (model 550) equipped with a microtip ( Fisher Scientific, Pittsburgh, PA). The sonication conditions were as follows: setting 4, 5 min total processing time, 15.0 s on-time, and 15.0 s off-time. Insoluble tissue debris was removed by centrifugation at 600 ×g and 4°C for 10 min. Liver homogenate protein concentration was determined utilizing the Bradford protein microassay (Bio-Rad) as described by the manufacturer, with use of Costar 96-well assay plates (Corning, Corning, NY) and the Synergy 2 microplate reader (BioTek Instruments, Winooski, VT).

Liver homogenate, serum, and biliary total bile acids were quantified using the Diazyme total bile acid diagnostic kit according to the manufacturer’s instructions, modified for use with the BioTek microplate reader. Total bile acid amount (nmol) was calculated using the following equation: total bile acid = [liver bile acid (nmol/liver) × liver weight (g)] + [serum bile acid (nmol/l × serum volume (liters)) + biliary bile acid (nmol/l × gallbladder bile volume (liters))]. Biliary cholesterol and phospholipid were quantified using the appropriate
Wako Chemicals diagnostic kit according to the manufacturer's instructions, again, modified for use with the BioTek microplate reader. Biliary lipid ratios of cholesterol to phospholipid, cholesterol to bile acid, phospholipid to bile acid, and phospholipid to (phospholipid + bicle acid); mol/mol and biliary cholesterol saturation index were determined as described elsewhere (9). Bile acids were isolated from gallbladder bile as described elsewhere (28). Individual bile acids were identified and quantified by HPLC essentially as described elsewhere (10, 28, 63), except a microBondapack 3.9 × 300-mm C18 column (Waters, Milford, MA) on a HPLC system equipped with an ELSD-LTI detector was used (Shimadzu Instruments, Columbia, MD). The mobile phase was 70% methanol-2 mM ammonium acetate (pH 5.4). Liver homogenate total cholesterol and free cholesterol were quantified using appropriate Wako Chemicals diagnostics kits as described above. Liver homogenate cholesteryl ester content was quantified by subtraction of the free cholesterol concentration from the total cholesterol concentration.

**Biliary secretion of HDL-cholesterol.** Mice were anesthetized as described above, and each mouse was injected via the hepatic portal vein with HDL containing small amounts of NBD-cholesterol or [3H]cholesterol label prepared as described previously (77). This labeling procedure did not significantly alter the lipid composition of the HDL (data not shown).

For determination of biliary secretion of HDL-derived NBD-cholesterol, 200 μI of HDL solution containing 4.4 mg protein/ml, 2.5 mM cholesterol (500 nmol injected), and 2.5 μM NBD-cholesterol (500 pmol injected) were injected. The injected mouse was kept under a warming lamp to maintain body temperature, and bile was collected at 0, 20, 40, 60, and 120 min postinjection. At 120 min postinjection, blood was collected by cardiac puncture and processed to serum, and the volume of serum collected was measured. The mouse was euthanized, and the liver was excised, briefly blotted dry, and weighed. A small (~0.1 g) piece of liver was homogenized, and the liver homogenate protein concentration was determined as described above. Lipids were extracted from the liver homogenate (1 mg of protein), 50 μl of serum, and 10 μl of bile at each time point and analyzed by thin-layer chromatography (TLC) as described elsewhere (6, 46). A standard curve using purified NBD-cholesterol was generated and analyzed by TLC as described above. After TLC, NBD-cholesterol was identified and quantified utilizing an Alpha Innotech ChemiImager system using FluorChem version 2.0 software (San Leandro, CA). The percentage of total NBD-cholesterol injected was determined as follows: [total picomoles in each sample × total picomoles of NBD-cholesterol injected (500 pmol)] × 100.

To determine biliary secretion of HDL-[3H]cholesterol, a second set of male WT mice was anesthetized as described above, and each mouse was injected with 200 μl of HDL solution containing 4.4 mg protein/ml, 2.5 mM cholesterol (500 nmol injected), and 0.25 μM [3H]cholesterol (50 pmol injected) prepared as described previously (77), except the NBD-cholesterol was replaced by [3H]cholesterol. These animals were treated as described above for the HDL-derived NBD-cholesterol-injected mice. Analysis of biliary, liver homogenate, and serum samples revealed no significant differences between NBD-cholesterol and [3H]cholesterol with respect to biliary secretion rate or tissue distribution of the injected HDL-cholesterol.

**Quantitative real-time RT-PCR.** Total RNA was isolated from liver and stored in RNAlater buffer using the RNeasy Mini Kit (Qiagen, Valencia, CA) according to the manufacturer's instructions. Expression patterns of mRNAs were evaluated utilizing the PRISM 7900 Sequence Detection System (Applied Biosystems) using TaqMan One Step PCR Master Mix Reagent kit and gene-specific assays for ABCG5, ABCG8, Cyp7A1, OATP1, OATP2, and NTCP. RNA measurements were analyzed utilizing ABI PRISM 7900 Sequence Detection System software as described in User Bulletin 2. All mRNA expression levels were normalized to 18S ribosomal RNA; data are presented in relative units, with WT mRNA level = 1.0.

**Western blotting.** Protein expression levels from liver homogenates were quantified as follows. A small (~0.1 g) piece of liver was homogenized in 10 mM Tris HCl (pH 7.4), 250 mM sucrose, and protease inhibitor cocktail (product no. 1836153, Roche Diagnostics, Indianapolis, IN) as described above for lipid analysis. After low-speed centrifugation to remove insoluble cellular debris, an aliquot of liver homogenate was added to one sample volume of 2× SDS-PAGE loading buffer [0.1 M Tris-HCl (pH 6.8), 0.3 M SDS, 0.6 M 2-mercaptoethanol, 1.6 M glycerol, and Coomassie Brilliant Blue G-250] and incubated at 95°C for 5 min. The samples were processed briefly in a mini-centrifuge and loaded onto a 12% polyacrylamide gel. After gel electrophoresis (constant voltage = 150 V), the proteins were transferred from the polyacrylamide gel to a nitrocellulose membrane (product no. 162-0112, Bio-Rad) on ice for 2 h (constant current = 500 mA). Complete protein transfer was confirmed by staining the nitrocellulose membrane with Ponceau S (product no. P7170, Sigma-Aldrich). To minimize nonspecific antibody interaction, the nitrocellulose membrane was incubated with TBST [10 mM Tris-HCl (pH 8.0), 150 mM NaCl, and 0.05% Tween 20] containing 3% gelatin for 1 h at room temperature prior to primary antibody exposure. The nitrocellulose membrane was then incubated with primary antibody in TBST + 1% gelatin overnight at room temperature with gentle shaking. The antibody solution was removed, and the nitrocellulose membrane was washed three times for 5 min each with TBST.

Alkaline phosphatase-conjugated secondary antibody in TBST + 1% gelatin was added to the nitrocellulose membrane, which was then incubated with gentle shaking at room temperature for 2 h. The secondary antibody was removed, and the nitrocellulose membrane was washed three times for 5 min each with TBST. The nitrocellulose membrane was incubated for 5 min at room temperature in alkaline phosphatase buffer [100 mM Tris-HCl (pH 9.0), 100 mM NaCl, and 5 mM MgCl2]. The alkaline phosphatase buffer was removed, and color development was initiated by addition of the alkaline phosphatase substrate 5-bromo-4-chloro-3-indolyl phosphate-nitro blue tetrazolium (Sigma-Aldrich) to the nitrocellulose membrane. After sufficient color development, the membrane was washed extensively with deionized water to stop the alkaline phosphatase reaction. Membrane densitometry analysis was performed utilizing Epson Perfection V700 Photo Scanner ImageJ image analysis software (version 1.48v, Wayne Rasband, National Institutes of Health, Bethesda, MD). SDS-PAGE gelloading control proteins (COX4, GAPDH, or β-actin) appropriate for the protein of interest, i.e., antibody cross-reactivity and protein molecular size, were used. Loss of L-FABP, SCP-2, and/or SCP-x had no effect on liver levels of any of the control proteins (data not shown). Protein quantification data are shown in relative units, with WT protein level = 1.0.

**Statistics.** Statistical analysis was performed by one-way analysis of variance (ANOVA) combined with the Newman-Keuls multiple-comparisons posttest (Prism version 3.03, GraphPad, San Diego, CA). All data passed Bartlett’s test for equal variances. Unless otherwise noted, values are means ± SE (n = 8 per group). Graphical analysis was accomplished using SigmaPlot 2002 for Windows version 8.02 (SPSS, Chicago, IL).

**RESULTS**

**Differential impacts of ablation of L-FABP, SCP-2/SCP-x, or both on bile acid levels in male mice.** While loss of SCP-2/SCP-x affects bile acid metabolism in mice, concomitant severalfold upregulation of L-FABP complicates interpretation (22, 36, 75). Therefore, the impact of ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) on hepatic, serum, biliary, and total bile acid levels was determined.

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Hepatic bile acid level was decreased significantly in mice lacking L-FABP (LKO and TKO) compared with WT animals (Fig. 1A). This effect was not observed in mice lacking SCP-2/SCP-x (DKO; Fig. 1A). While serum bile acid levels followed a similar pattern, this trend did not approach statistical significance (Fig. 1B).

In contrast, loss of L-FABP or SCP-2/SCP-x oppositely impacted biliary bile acid level. L-FABP gene ablation (LKO) significantly increased biliary bile acid by 40% (Fig. 1C), while SCP-2/SCP-x gene ablation (DKO) decreased biliary bile acid concentration (Fig. 1C). Ablation of L-FABP in SCP-2/SCP-x null mice (TKO) reversed this decrease; the net result was that biliary bile acid concentration in TKO mice was similar to that in WT mice (Fig. 1C).

When the total bile acid in serum, liver, and biliary bile was combined, a pattern similar to that of biliary bile acid was observed. LKO mice had significantly more total bile acid (Fig. 1D); however, ablation of SCP-2/SCP-x (DKO) resulted in lowered total bile acid (Fig. 1D). Ablation of L-FABP in DKO mice resulted in no net change in total amount of bile acid in TKO animals relative to WT mice (Fig. 1D).

Thus, loss of L-FABP increased biliary bile acid levels at the expense of hepatic bile acid levels. In contrast, loss of SCP-2/SCP-x did not affect hepatic and serum bile acid levels, while biliary and total bile acid levels were decreased. The net effect of ablation of both L-FABP and SCP-2/SCP-x genes was a decrease in hepatic bile acid concentration without a significant decrease in serum, biliary, or total bile acid levels.
Effect of ablation of L-FABP, SCP-2/SCP-x, or both on biliary lipid composition. Since L-FABP and SCP-2/SCP-x gene products differentially impacted biliary secretion of bile acid and HDL-derived cholesterol, it was important to determine their effects on biliary phospholipid, cholesterol, and cholesterol saturation indexes.

Loss of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) did not significantly alter biliary phospholipid levels (Fig. 1E). In contrast, loss of L-FABP and/or SCP-2/SCP-x function resulted in a 40% decrease in biliary cholesterol, although this effect was somewhat lessened in TKO mice (Fig. 1F).

The combined effect of decreased biliary bile acid (Fig. 1C) concomitant with decreased biliary cholesterol (Fig. 1F) but unaltered biliary phospholipid (Fig. 1E) levels in L-FABP null (LKO) male mice was a decrease in the molar ratios of cholesterol to phospholipid (Fig. 1G), cholesterol to bile acid (Fig. 1H), and phospholipid to bile acid (Fig. 1I) in these animals compared with their WT counterparts. In contrast, SCP-2/SCP-x gene ablation decreased only the biliary cholesterol-to-phospholipid ratio (Fig. 1G), but not the ratio of cholesterol to bile acid (Fig. 1H) or phospholipid to bile acid (Fig. 1I). Ablation of both the L-FABP and SCP-2/SCP-x (TKO) genes decreased the biliary cholesterol-to-phospholipid (Fig. 1G) and cholesterol-to-bile acid (Fig. 1H) ratios, but not the phospholipid-to-bile acid ratio (Fig. 1F).

On the basis of the above-described biliary bile acid, cholesterol, and phospholipid determinations, the biliary cholesterol saturation index (an indicator of the amount of cholesterol that can be solubilized in bile) was calculated as described elsewhere (9). Loss of L-FABP alone reduced the biliary cholesterol saturation index by 17% (Fig. 1J). By contrast, DKO or TKO animals exhibited minor, if any, changes in biliary lipid ratios, resulting in no net effect on the cholesterol saturation index in these mice compared with WT animals (Fig. 1J).

Taken together, these data indicate that L-FABP, rather than SCP-2/SCP-x gene products, decreased the biliary cholesterol saturation index to reduce the ability of biliary bile from L-FABP gene-ablated mice to solubilize cholesterol.

Impact of LKO, DKO, and TKO on composition of biliary bile acids in male mice. Biliary bile acid composition determines the hydrophilic/hydrophobic balance (hydrophobicity index) of mixed bile salt solutions, which is an indicator of the ability of biliary bile acids to solubilize cholesterol (26). L-FABP inhibits, while SCP-2/SCP-x gene products stimulate, several enzymatic steps of bile acid formation (37, 68, 70). This suggests that ablation of one or both of these genes may also alter the biliary bile acid composition and the hydrophobicity index. Therefore, the impact of ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) on the composition of biliary bile acids was determined.

Bile of all mouse strains contained at least nine different species of bile acids (Fig. 2A). The majority (>75%) of these biliary bile acids were taurine-conjugated, with the major species being T-MCA and, to a lesser extent, T-CA. The unconjugated population (~25%) of biliary bile acids comprised small amounts of β-MCA > α-MCA >> CA.

Although ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) did not alter the overall ratio of conjugated to unconjugated bile acids (Fig. 2B), the biliary ratio of unconjugated β-MCA to unconjugated α-MCA was significantly increased (Fig. 2C). β-MCA has a higher hydrophobicity index than α-MCA (26). L-FABP gene ablation alone decreased biliary T-MCA and α-MCA by 25% and 80%, respectively (Fig. 2A). Concomitantly, ablation of only L-FABP increased biliary β-MCA and T-CA by 35% and 75%, respectively (Fig. 2A). By contrast, in biliary bile from DKO and TKO male mice, T-MCA and T-CA levels were unchanged (Fig. 2A) but T-LCA increased 2- to 2.7-fold and α-MCA decreased 60–65% (Fig. 2A).

The combined effect of these alterations in biliary bile acid composition was to significantly increase (i.e., make less negative) the overall biliary bile acid hydrophobicity index in mice ablated in L-FABP, SCP-2/SCP-x, or both (Fig. 2D). Ablation of L-FABP alone or SCP-2/SCP-x alone increased the biliary bile acid hydrophobicity index by 28% and 22%, respectively, compared with WT mice (Fig. 2D). Ablation of both genes induced a similar pattern (Fig. 2D). The increased biliary bile acid hydrophobicity index, especially in L-FABP or SCP-2/SCP-x gene-ablated mice, suggests an increased ability to solubilize cholesterol.

SCP-2/SCP-x, but not L-FABP, gene ablation increases hepatic cholesterol. Because of the increased bile acid hydrophobicity (Fig. 2D), altered cholesterol absorption efficiency may have contributed to modifications in hepatic sterol levels. Therefore, hepatic levels of cholesterol, cholesteryl ester, and total cholesterol were determined in LKO, DKO, and TKO mice.

Ablation of L-FABP alone had no effect on hepatic total cholesterol, unesterified cholesterol, or cholesteryl ester (Fig. 3). In contrast, loss of SCP-2/SCP-x (DKO and TKO) significantly increased hepatic total cholesterol (Fig. 3A), primarily cholesteryl ester in DKO and unesterified cholesterol and cholesteryl ester in TKO (Fig. 3, B and C).

Thus, increased bile acid hydrophobicity index, observed in all three genetically altered mouse strains (Fig. 2D), may in part contribute to increased hepatic cholesterol levels in mice devoid of SCP-2/SCP-x (DKO and TKO), but not in LKO mice, in which hepatic cholesterol level was not significantly altered.

L-FABP, but not SCP-2/SCP-x, gene ablation decreases biliary secretion of HDL-cholesterol. Since L-FABP and SCP-2 bind cholesterol (44, 69, 71), the possibility that L-FABP and/or SCP-2/SCP-x gene ablation may impact biliary secretion of HDL-derived cholesterol was examined. To avoid having to use HDL loaded with radiolabeled cholesterol for measurement of biliary HDL-cholesterol excretion, a new HDL-derived NBD-cholesterol excretion assay was developed for fluorescence detection. To validate this assay, male WT mice were first injected with a bolus of HDL containing [3H]cholesterol or NBD-cholesterol via the hepatic portal vein, as described in Experimental Procedures. Bile was collected as a function of time postinjection, and distribution of [3H]cholesterol and NBD-cholesterol in liver, serum, and bile was determined at the end of the experiment, also as described in Experimental Procedures.

HDL-derived NBD-cholesterol was rapidly cleared from serum, taken up by mouse liver, and appeared in bile with kinetics similar to HDL-derived [3H]cholesterol (Fig. 4 and Fig. 4, inset). The kinetics of biliary HDL-derived [3H]choles-
terol and NBD-cholesterol were similar to those previously obtained for HDL-derived $^{14}$C-free cholesterol (Fig. 4, inset) (29). HDL-derived NBD-cholesterol was unesterified in serum, liver, and bile 1 h after intravenous injection, very similar to HDL-derived $[^3]$H-cholesterol (Fig. 4) and $^{14}$C-free cholesterol (Fig. 4, inset) (29). Rapid isolation of HDL from serum (29) showed 10% of HDL-derived NBD-cholesterol transfer to non-HDL lipoproteins, and clearance from the latter was negligible (data not shown), also similar to HDL-derived $^{14}$C-free cholesterol (29). These data suggest that in vivo clearance, hepatic uptake, and biliary secretion of HDL-derived NBD-cholesterol reflected these same characteristics of HDL-derived radiolabeled cholesterol, indicating that NBD-cholesterol is a suitable probe for monitoring biliary clearance of HDL-cholesterol.

The loss of L-FABP (LKO or TKO) markedly decreased the rate of biliary secretion of HDL-derived NBD-cholesterol by ~85% (Fig. 5), with maximal decreased appearance in biliary bile occurring by 60 min (Fig. 5). As a result, the loss of L-FABP (LKO or TKO) resulted in a cumulative ~85% decrease in total appearance of HDL-derived NBD-cholesterol in the biliary bile (Fig. 6A). In contrast, SCP-2/SCP-x gene ablation (DKO) had no significant effect on the rate of appearance (Fig. 5) and cumulative total appearance (Fig. 6A) of HDL-derived NBD-cholesterol in the bile of these mice. Ablation of L-FABP, SCP-2/SCP-x, or both had no significant effect on biliary volume (data not shown) or on the amount of esterified NBD-cholesterol produced during the experiment [<5% of NBD cholesterol converted to NBD-cholesteryl ester (data not shown)].

L-FABP ablation (LKO or TKO) adversely impacted not only biliary excretion, but also hepatic uptake of HDL-derived NBD-cholesterol. Loss of L-FABP (LKO and TKO) reduced hepatic uptake of HDL-derived NBD-cholesterol by 76% and 60%, respectively (Fig. 5B). Similarly, loss of L-FABP (LKO or TKO) decreased HDL-derived NBD-cholesterol remaining in the serum by 54% and 60%, respectively (Fig. 6C). By contrast, loss of SCP-2/SCP-x (DKO) had no significant effect on hepatic uptake of HDL-derived NBD-cholesterol (Fig. 6B) or removal of HDL-derived NBD-cholesterol from serum of these male mice compared with WT animals (Fig. 6C), suggesting removal by nonhepatic tissues.
Taken together, these data demonstrate a greater impact of L-FABP than SCP-2/SCP-x gene products in promoting hepatic uptake and biliary excretion of HDL-derived cholesterol. Effect of ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) on hepatic expression of the HDL receptor SR-B1. Since HDL cholesterol represents the major source of biliary cholesterol, the potential of concomitant altered expression of the HDL receptor SR-B1 levels, which instead were unaltered.

Effect of ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) on key hepatic proteins involved in cholesterol esterification and bile acid metabolism. Since hepatic and/or biliary bile acid levels were significantly altered in LKO, DKO, and TKO mice, the possibility that these alterations were due to inhibition of the bile acid synthesis pathway was examined.

Loss of L-FABP (LKO or TKO) resulted in 30% increased ACAT2, a key enzyme diverting cholesterol from bile acid synthesis toward storage as cholesteryl esters (Fig. 7B). Loss of L-FABP (LKO or TKO) also resulted in 2.5- to 3.5-fold induction of Cyp7A1 mRNA (Fig. 7C), 35–70% increase in 3\halpha-HSD (Fig. 7E), and 1.9- to 2.3-fold increase in LXR\halpha (Fig. 7G). By contrast, SCP-2/SCP-x gene ablation (DKO) had no NBD-cholesterol uptake due to reduced liver SR-B1 levels, which instead were unaltered.
effect on the liver concentration of ACAT2 or LXRα (Fig. 7, B and G) but did increase Cyp7A1 mRNA by 2.8-fold (Fig. 7C). Among the mouse strains examined, DKO males exhibited the highest levels of Cyp7A1 (2.7-fold induction; Fig. 7D), 3α-HSD (1.9-fold induction; Fig. 7E), PPARα (3.3-fold induction; Fig. 7F), and FXR (1.9-fold induction; Fig. 7I). DKO mice also had increased (3.2-fold induction) liver levels of SHP (Fig. 7H). Loss of L-FABP/SCP-2/SCP-x (TKO) resulted in a combination of these effects, i.e., increased ACAT2, Cyp7A1 mRNA, 3α-HSD, PPARα, LXRα, SHP, and FXR (Fig. 7, B, C, and E–I). In summary, while loss of L-FABP, SCP-2/SCP-x, or both modestly increased ACAT2 to divert some cholesterol toward storage, several enzymes in bile acid synthesis were concomitantly upregulated: Cyp7A1 (the rate-limiting enzyme in bile acid formation), 3α-HSD, and, in the case of DKO and TKO mice, Cyp27A1 (the key enzyme initiating the alternate pathway of bile acid synthesis).

Effect of ablation of L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) on key hepatic proteins involved in transport of cholesterol, bile acid, and phospholipid into bile. Since hepatic and/or biliary bile acid levels were significantly altered in LKO, DKO, and TKO mice, the concentrations of proteins involved in the hepatobiliary transport of cholesterol, bile acid, and phospholipid were determined.

L-FABP gene ablation (LKO or TKO) resulted in a 2.2- to 2.7-fold and 30% induction of ABCG5 (hepatic cholesterol efflux) mRNA and MDR3 (hepatic phospholipid efflux) protein, respectively (Fig. 8, A and C). In addition, loss of L-FABP (LKO and/or TKO) increased by two- to threefold the level of the major hepatic basolateral membrane transport proteins involved in the uptake/recovery of bile from plasma, including OATP2 mRNA and/or NTCP mRNA (Fig. 8, E and F). Finally, loss of L-FABP only (LKO) resulted in 73% and 22% induction of the canalicular bile salt transporters BSEP and MRP2, respectively (Fig. 8, G and H). In contrast, SCP-2/SCP-x gene ablation (DKO) resulted in offsetting changes in hepatic basolateral membrane transport proteins involved in the uptake/recovery of bile from plasma, i.e., a 3.4-fold induction of OATP1 mRNA (Fig. 8D), but 60% and 80% decrease in OATP2 mRNA (Fig. 8E) and NTCP mRNA (Fig. 8F), respectively. Loss of SCP-2/SCP-x had no effect on BSEP levels; however, MRP2 increased 50% in these DKO mice (Fig. 8, G and H). L-FABP and SCP-2/SCP-x gene ablation (TKO) had no effect on these canalicular bile salt transporters (Fig. 8, G and H).

Overall, loss of L-FABP increased expression of proteins involved in excretion of cholesterol and phospholipid into bile and also increased several basolateral transporters involved in recovery of bile acids from the plasma. In contrast, SCP-2/SCP-x gene ablation had little net effect on expression of these proteins.

DISCUSSION

Resolving the components/pathways contributing to biliary bile formation is critically important to understanding cholesterol homeostasis, since canalicular bile acid secretion is the rate-limiting step driving biliary cholesterol secretion, the major pathway for net cholesterol elimination in mammals (60, 64, 80). Great advances have been made in identifying the enzymes, proteins, and receptors involved in basolateral uptake of bile acid and HDL-cholesterol for biliary secretion (11, 60, 80). In contrast, very little is known about the cytosolic proteins that transfer the poorly aqueous soluble bile acids across the cytoplasm for secretion into bile (1, 24, 42, 50, 64, 80). Similarly, although >95% of biliary cholesterol is derived from HDL-cholesterol taken up via the SR-B1 pathway (11, 29, 62, 91), almost nothing is known about the cytosolic proteins involved in transporting these even more poorly soluble molecules across the cytoplasm for secretion into bile (11, 18). Although in vitro ligand-binding/transfer assays outlined in the introduction suggest that L-FABP, SCP-2, and SCP-x may contribute to this function, their physiological significance is not completely clear. Therefore, male mice lacking L-FABP (LKO), SCP-2/SCP-x (DKO), or both (TKO) genes were generated to investigate the physiological role(s) of these
proteins in biliary bile acid and cholesterol formation. The data described here describe several new insights indicating important, but different, roles for L-FABP and SCP-2 in biliary cholesterol and bile acid homeostasis in these animals.

L-FABP played a major role in retaining bile acids in liver. Loss of L-FABP markedly increased (LKO) or maintained (TKO) biliary bile acid, maintained (LKO or TKO) serum levels, and decreased levels in the liver. The increase in biliary bile acid in male LKO mice was consistent with an earlier study with male LKO mice (45). However, in the latter study the hepatic and serum levels of bile acids were also increased, rather than decreased or unaltered, as shown in the current study, a discrepancy likely associated with the earlier LKO mice being only a N2 backcross generation, i.e., still on a mixed background (75% congenic). In contrast, the LKO mice used in the current study were backcrossed to congenic 99% congenic. In LKO mice, this redistribution was not due to reduced total bile acid among these tissues, since

Fig. 7. Hepatic concentrations of proteins involved in cholesterol uptake, esterification, and conversion into bile acid. A, B, and D–I: aliquots of liver homogenate proteins [scavenger receptor class B type 1 (SR-B1), acetyl-CoA acetyltransferase-2 (ACAT2), sterol 27-hydroxylase (Cyp27A1), 3α-hydroxysteroid dehydrogenase (3α-HSD), peroxisome proliferator-activated receptor-α (PPARα), liver x receptor-α (LXRα), short heterodimer partner (SHP), and farnesoid x receptor (FXR)] were examined by SDS-PAGE and subsequent Western blot analysis. Representative Western blots and analysis of multiple Western blots are shown for the protein of interest and the gel-loading control protein [cytochrome c oxidase subunit 4 (COX4), GAPDH, or β-actin]. C: quantitative real-time PCR to determine abundance of hepatic Cyp7A1 mRNA. Values [relative to WT (WT = 1)] are means ± SE (n = 8 mice per group). Statistically similar values (ANOVA) are denoted by the same lowercase letter.

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total bile acid was increased. Absence of L-FABP in TKO mice also resulted in reduced hepatic bile acid levels; however, biliary bile acid and total bile acid levels were unchanged. In WT mice, L-FABP normally represents the major hepatic BABP in liver cytosol (13–16, 25, 35, 48, 79). L-FABP also increases hepatic cytosolic binding capacity of another ligand, long-chain fatty acids (LCFAs), to sequester/retain/enhance uptake of LCFAs in the cytoplasm in cultured primary hepatocytes and liver in vivo (4, 46, 49, 76). By exhibiting highest affinity for the more hydrophobic hepatotoxic bile acids than the more hydroxylated less hepatotoxic bile acids, L-FABP may exert a protective effect on hepatocytes (78). L-FABP also bound other oxidized lipids to protect hepatic cells from oxidative stress (58, 85, 86, 89, 90). L-FABP inhibits microsomal sulfation of select bile acids in vitro (70), is essential for bile acid uptake and intracellular transport as evidenced by photoaffinity-labeled bile acid cross-linking studies (15), and decreases bile acid distribution into some intracellular compartments (82). Thus, by binding bile acids in the cytoplasm, L-FABP may act to retain bile acids within the hepatocytes and decrease their excretion into bile via export pumps that concentrate bile acids >1,000-fold in bile compared with hepatocytes (43). Conversely, loss of L-FABP may be more permissive for bile acid canicular excretion into bile. It is important to note that loss of L-FABP did not reduce hepatic bile acid synthetic capacity but, rather, increased transcription of key enzymes in bile acid synthesis. The net effect of loss of L-FABP was to increase transcription of Cyp7A1 (the rate-limiting enzyme in bile acid synthesis) as well as 3α-HSD. Thus the loss of L-FABP-induced decrease in bile acid in liver concomitant with increased bile acid in bile was not likely due to inhibition of bile acid synthesis. Finally, the loss of L-FABP also differentially regulated the expression of basolateral and canalicular membrane bile acid transporters. L-FABP ablation increased expression of OATP2 and NTCP but did not alter OATP1 expression, suggesting increased reuptake of bile acid from the serum. Indeed, loss of L-FABP (LKO or TKO) tended to decrease serum bile acid levels, but this trend did not achieve statistical significance. In contrast, loss of L-FABP increased expression of BSEP and MRP2, canalicular transport proteins involved in bile acid secretion into bile. Combined with the loss of cytosolic bile acid-binding capacity in mice devoid of L-FABP, the increased expression of canalicular bile acid transporters would account for the increased bile acid secretion across the canalicul membrane into the gallbladder bile.

SCP-2/SCP-x oppositely contributed to biliary bile acid level. In contrast to L-FABP retaining bile acid in the liver, SCP-2/SCP-x enhanced bile acid distribution into bile. Loss of SCP-2/SCP-x markedly decreased biliary bile acid and, in addition, reduced the combined total bile acid in liver, serum, and gallbladder bile. These observations were consistent with earlier findings that 1) SCP-2 stimulates liver microsomal cholesterol 7α-hydroxylase (the rate-limiting enzyme in hepatic bile acid synthesis) while SCP-x is the only known peroxisomal branched-chain ketothiolase for oxidizing cholesterol’s side chain required for bile acid synthesis (37, 68); 2) SCP-2 overexpression increases, while ablation decreases, bile

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**Fig. 8. Changes in key bile acid transporter concentration in L-FABP/SCP-2/SCP-x gene-ablated mice. A, B, and D–F: relative mRNA concentrations (WT = 1) of ATP-binding cassette transporters G5 and G8 (ABCG5 and ABCG8), organic anion transporting polypeptides 1 and 2 (OATP1 and OATP2), and sodium taurocholate cotransporting polypeptide (NTCP) were determined by quantitative real-time PCR. C, G, and H: relative protein concentrations (WT = 1) of multidrug-resistance-3 P-glycoprotein (MDR3), bile salt export pump (BSEP), and multidrug-resistance-associated protein 2 (MRP2) were determined by Western blot analysis. Representative Western blots and analysis of multiple Western blots are shown for the protein of interest and the gel-loading control protein (COX4 or GAPDH). Values are means ± SE (n = 8 mice per group). Statistically similar values (ANOVA) are denoted by the same lowercase letter.**
acid synthesis and biliary bile acid secretion (2, 22, 36, 59); and 3) SCP-2/SCP-x gene ablation elicits compensatory up-regulation of L-FABP (22, 36, 69, 75).

Loss of SCP-2/SCP-x did not reduce hepatic bile acid synthetic capacity. Normally, SCP-2 binds and facilitates intermembrane transfer not only of cholesterol (44, 47, 71), but also oxysterols such as 7α-hydroxycholesterol (81). 7α-Hydroxycholesterol is a potent inducer of LXRα transcription of Cyp7A1 and other enzymes in bile acid synthesis (60, 64). While SCP-2 also binds cholesterol hydroperoxides, it facilitates, rather than prevents, the toxic effects of these hydroperoxides (39–41, 84). Analogous to the effects of L-FABP-binding bile acids in the cytosol to reduce bile acid distribution to the nucleus (82), by binding oxysterols the SCP-2 may sequester these ligands in the cytosol and peroxisomes to decrease their availability in the nucleus. This may explain in part why loss of SCP-2/SCP-x increased expression of Cyp7A1 and Cyp27A1, despite unaltered levels of LXRα. Finally, the loss of SCP-2/SCP-x also differentially regulated expression of basolateral and canalicular membrane bile acid transporters. SCP-2/SCP-x ablation increased expression of OATP1 but decreased expression of OATP2 and NTCP, suggesting no net effect on reuptake of bile acid from the serum. Loss of SCP-2/SCP-x also altered expression of canalicular transport proteins involved in bile acid secretion into bile. Although MRP2 expression was increased in DKO mice, the absence of SCP-2/SCP-x had no effect on BSEP expression.

Loss of L-FABP and, to a lesser extent, SCP-2/SCP-x altered the composition of biliary bile acids without altering the conjugated-to-unconjugated ratio. L-FABP gene ablation significantly decreased the percentage of T-MCA and α-MCA while increasing the percentage of T-CA and β-MCA. In contrast, as shown here, loss of SCP-2/SCP-x primarily increased T-LCA but decreased α-MCA, while SCP-2 overexpression alone increased β-MCA, in rat hepatocytes (59). As a result, loss of L-FABP and, to a lesser extent, loss of SCP-2/SCP-x reduced the ratio of β-MCA to α-MCA. Since CA s are agonists and MCAs are antagonists of FXR (65), the decreased MCAs concomitant with increased CAs may contribute in part to the increase in FXR-regulated expression of Cyp7A1. Finally, loss of L-FABP or SCP-2/SCP-x also increased the overall hydrophobicity index of the bile acids, suggesting increased ability to solubilize cholesterol. The above-observed effects of loss of L-FABP and SCP-2/SCP-x on biliary bile acid percent composition may be attributed in part to the very different ligand-binding capacity and subcellular compartmentalization of these proteins. 1) L-FABP binds/transfers cholesterol, the substrate for bile acid synthesis, and is optimally localized (in cytosol, endoplasmic reticulum, and mitochondria) for transfer of cholesterol and its metabolites between extraperoxisomal sites (3, 8, 44). In contrast, while SCP-2 and SCP-x also bind/transfer cholesterol, the majority of SCP-2 and SCP-x is localized in the peroxisomal matrix, where they appear to facilitate oxidation of the cholesterol branched side chain for bile acid synthesis (23, 38, 44, 71, 72). 2) L-FABP preferentially binds conjugated bile acids with affinities ranging ~100-fold (e.g., T-LCA > T-cholate > T-choleate > Τ-CA > CA), while there is no evidence that SCP-2 binds bile acids (78). 3) While neither L-FABP nor SCP-2 exhibits enzymatic activity, SCP-x is the only known peroxisomal branched-chain ketohiolase for oxidizing the cholesterol side chain required for bile acid synthesis (68). Nevertheless, L-FABP and SCP-2 can impact the activity of enzymes in bile acid synthesis. For example, rat L-FABP inhibits liver microsomal sulfation of glycolithocholic acid in vitro (70). In contrast, SCP-2 stimulates liver microsomal cholesterol 7α-hydroxylase (the rate-limiting enzyme in hepatic bile acid synthesis) in vitro (37). Whether L-FABP and SCP-2 binding of cholesterol and/or intermediates in bile acid synthesis affects activities of other enzymes in the bile acid synthesis pathway remains to be resolved. L-FABP and SCP-2 may inhibit or enhance delivery of bound substrates to these enzymes, depending on the molar ratio of ligand to L-FABP or SCP-2, as has been shown for LCFAs and LCFA-CoAs (30–34, 67).

L-FABP expression regulated hepatic uptake of HDL-derived cholesterol. WT mouse liver cleared ~80% of injected HDL-derived cholesterol. Loss of L-FABP (LKO or TKO) reduced hepatic clearance of HDL-derived NBD-cholesterol from serum. LKO and TKO also lowered the percentage of injected HDL-derived cholesterol remaining in serum. Taken together, these findings suggest that loss of L-FABP shifted deposition of HDL-derived cholesterol away from liver and toward other tissues, where the HDL receptor SR-B1 is also expressed. Indeed, the HDL-receptor SR-B1 is abundant not only in liver, but also in steroidogenic tissues (adrenal, testis, and ovary), and is expressed in cells of the arterial wall (endothelial cells, smooth muscle cells, and macrophages) (29, 61, 83). Since SR-B1 facilitates bidirectional transfer of HDL-cholesterol not only to/from hepatocytes, but also other cells such as macrophages and fibroblasts (7, 19, 75, 77, 83), reduced appearance of HDL-derived NBD-cholesterol in liver and serum apparently resulted in deposition in multiple other tissues. Likely tissues for deposition were those expressing high levels of SCP-2 (adrenal, testis, ovary, and macrophages) (23, 27). SCP-2 overexpression is known to enhance HDL-cholesterol uptake in cultured primary hepatocytes and facilitate cholesterol retention by inhibiting cholesterol efflux to HDL (7, 75, 77), and loss of SCP-2/SCP-x (DKO) was expected to reduce hepatic clearance of HDL-derived NBD-cholesterol. This was due to concomitant upregulation of L-FABP in liver and hepatocytes of DKO mice, which compensated for the loss of SCP-2/SCP-x (22, 69, 77).

L-FABP, but not SCP-2/SCP-x, markedly impacted biliary secretion of HDL-derived cholesterol. While >95% of biliary cholesterol is derived from HDL-cholesterol (11, 29, 62, 91), it was previously unknown how this very poorly soluble molecule was transferred across the cytoplasm to the bile canaliculus for secretion into bile (11, 18). The finding that loss of L-FABP (LKO or TKO), but not SCP-2/SCP-x (DKO) alone, decreased hepatic uptake and biliary secretion of HDL-derived NBD-cholesterol establishes for the first time that L-FABP, rather than SCP-2/SCP-x, is the key protein serving this function. The decreased hepatic uptake of HDL-derived NBD-cholesterol in LKO or TKO mice was not due to a significant alteration in hepatic expression of SR-B1. Similarly, the reduced biliary secretion of HDL-derived cholesterol in LKO and TKO mice was not due to reduced level of ABCG5/ABCG8, the canalicular transporter mediating cholesterol efflux/secretion across the canalicular membrane into bile. Instead, loss of L-FABP increased expression of LXRα, known to stimulate transcription of ABCG5/ABCG8 (60, 64) and
increase expression of ABCG5 and trended to increase ABCG8. L-FABP’s role as a carrier/cytosolic transporter of HDL-derived cholesterol to the canalicular membrane for excretion into bile was supported by earlier studies. Fischer et al. (17) and Martin et al. (44) reported that L-FABP binds cholesterol in vitro. Frolov et al. (20, 21) and Nemez and Schroeder (55) demonstrated that L-FABP enhances intermembrane sterol transfer in vitro and in L-FABP-overexpressing cells. This finding was analogous to L-FABP overexpression increasing L-FABP ablation decreasing the diffusion/transport of another L-FABP ligand (NBD-stearic acid) in cultured cells and primary hepatocytes (4, 48, 52, 87). Storey et al. (75, 77) showed that L-FABP upregulation in SCP-2/SCP-x gene-ablated mouse primary hepatocytes increased HDL-cholesterol uptake while decreasing cholesterol efflux to HDL. Thus it was not surprising that SCP-2/SCP-x gene ablation did not alter biliary secretion of HDL-derived cholesterol, despite the fact that SCP-2 binds cholesterol (44, 47, 69, 71), and a variety of in vitro and cultured cell studies suggest a potential role (20, 21, 51, 53). Interestingly, adrenoviral overexpression of SCP-2 and SCP-2 antisense treatment oppositely affected de novo cholesterol synthesis and biliary secretion of de novo synthesized cholesterol, a relatively small (~5%) contributor to biliary cholesterol in vivo (2, 57).

L-FABP played a larger role than SCP-2/SCP-x in regulating biliary indexes of cholesterol solubility. LKO decreased most indexes of cholesterol solubility (cholesterol saturation index and cholesterol-to-phospholipid, cholesterol-to-bile acid, and phospholipid-to-bile acid ratio) while increasing only the bile acid compositional hydrophobicity index. These findings suggest that L-FABP gene ablation might significantly decrease cholesterol solubility in biliary bile, consistent with the decrease in cholesterol mass. Studies with another L-FABP gene-ablated mouse also showed increased susceptibility to lithogenic diet-induced gallstone formation (88).

In summary, unlike nonmammalian vertebrates, the mammalian liver does not contain the hepatic BABP (L-BABP) (24, 50). In contrast, mammalian liver does contain high levels of L-FABP, a protein that also binds bile acids, although only at a single site and with lower affinity than L-BABP (13–16, 25, 35, 79). These findings, together with a limited amount of other in vitro data, suggested that L-FABP may fulfill the role of L-BABP in bile acid metabolism and secretion. The work presented here utilized mice individually ablated in L-FABP, SCP-2/SCP-x, or both to provide significant new insights into our understanding of the physiological roles of L-FABP, as well as SCP-2, in hepatic bile acid metabolism. L-FABP preferentially facilitated hepatic retention of bile acids at the expense of biliary bile acids. Conversely, SCP-2/SCP-x preferentially facilitated bile acid excretion into gallbladder bile. L-FABP, more so than SCP-2/SCP-x, impacted the bile acid composition and indexes contributing to cholesterol solubility in gallbladder bile. Finally, L-FABP, but not SCP-2/SCP-x, facilitated biliary secretion of HDL-derived cholesterol. These results suggest important, but different, roles for L-FABP and SCP-2/SCP-x in regulating hepatic biliary acid and cholesterol homeostasis.

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**DISCLOSURES**

No conflicts of interest, financial or otherwise, are declared by the authors.

**AUTHOR CONTRIBUTIONS**

G.G.M. and B.P.A. contributed to the design of the research; B.P.A., K.K.L., D.L., S.M.S., and P.N.H. performed the experiments; G.G.M., B.P.A., K.K.L., D.L., S.M.S., and P.N.H. analyzed the data; G.G.M., K.K.L., A.B.K., and F.S. interpreted the results of the experiments; G.G.M. drafted the manuscript; G.G.M., B.P.A., K.K.L., D.L., S.M.S., P.N.H., A.B.K., and F.S. approved the final version of the manuscript; B.P.A., P.N.H., A.B.K., and F.S. are responsible for conception and design of the research; P.N.H., A.B.K., and F.S. edited and revised the manuscript.

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