The mechanism of increased biliary lipid secretion in mice with genetic inactivation of bile salt export pump

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Bile salts (BS) play important roles in stimulating bile formation, in facilitating absorption of dietary lipids and fat-soluble vitamins, and in regulating gene expression as ligands of the farnesoid X-activated receptor FXR and of the plasma membrane-bound bile acid receptor TGR5 (25, 38, 49, 50). Hepatobiliary BS transport across the canalicul membrane is mediated by the bile salt export pump (BSEP) and the other canalicular transporter proteins. BSEP is largely responsible for the accumulation of BS within hepatocytes, liver injury, and cholestasis (19).

In contrast to PFIC2 patients, Bsep-deficient mice display only a mild nonprogressive cholestasis (9, 25, 49, 50). The relatively mild phenotype seems related to the relatively hydrophilic BS composition in mice, since feeding low dosages of the hydrophilic BS cholate induces a severely cholestatic, and in male mice even fatal, phenotype (49). Similar to human PFIC2 patients, Bsep−/− mice have strongly reduced canalicular BS secretion of BS, especially of cholic acid conjugates and specific dihydroxy BS (33, 49, 50). Secretion of more hydrophilic BS, such as the muricholates, is less affected. Wang et al. provided evidence that P-glycoproteins can partially compensate for canalicular BS secretion in Bsep−/− mice (48).

MATERIALS AND METHODS

Animals. Breeder pairs of mice homozygous for disruption of the BS export pump gene Bsep (Bsep−/−) and littermate wild-type mice (Bsep+/+) were kindly provided by V. Ling (British Columbia Cancer Research Center, Vancouver, BC, Canada). The mice were inbred to a C57BL/6J background for more than 10 generations. We generated a local breeding colony, based on heterozygote breeding. Mice were housed in a light-controlled (lights on 6:00 A.M.–6:00 P.M.) and temperature-controlled facility and received standard lab chow (Hope Farms, Woerden, The Netherlands). Food and water were available ad libitum. All experimental protocols were approved by the Ethics Committee for Animal Experiments of the University of Groningen (Groningen, the Netherlands).

Experimental procedures. Bsep−/− and Bsep+/+ mice (n = 7, 4 males; and, n = 10, 4 males; respectively; age 2–5 mo) were anesthetized by intraperitoneal injection with Hypnorm (fentanyl/ fluanisone, 1 ml/kg) and diazepam (10 mg/kg). Bile was collected by...
cannulation of the gall bladder. Following 5 min of equilibration, bile was collected for an additional 90 min in 30-min fractions, representing the basal (unstimulated) bile production. Whereas concentrations of most BS species are greatly reduced in Bsep−/− mice, β-muricholic acid is present at similar concentrations in bile of Bsep−/− and Bsep+/+ mice (50). We therefore decided to use the preserved secretion of β-muricholic acid to analyze the mechanism of BS-induced biliary lipid secretion in Bsep−/− mice. Hereto, after the initial 90-min (unstimulated) bile collection, TβMCA (70 mM in phosphate-buffered saline) was infused via the jugular vein in stepwise increasing dosages over a 2.5-h period. TβMCA was obtained from S. Tazuma (Hiroshima University, Hiroshima, Japan). TβMCA infusion rates were 150, 300, and 450 nmol/min, each for 30 min, followed by 600 nmol/min for 60 min. Infusion rates and concentrations of TβMCA were similar to previously reported infusion experiments with taurolithocholic acid (Pemr−/− mice) (44) and similar to others with other BS as described in literature (13, 31, 35). Body temperature was stabilized during bile collection using a humidified incubator. Bile flow was determined gravimetrically, assuming a density of 1 g/ml for bile. Previously, we demonstrated that the bile salt-independent fraction (BSIF) of bile flow correlated negatively with the ratio of either biliary PL or CH to BS: the higher the BSIF, the lower the lipid-to-BS ratio (46). Bile was stored at −20°C until analysis of BS, PL, and CH concentrations. At the end of the infusion period, animals were killed by cardiac puncture. For determination of mRNA (RT-PCR) and protein (Western blot) expression levels of relevant canaliculal transport proteins (Mdr2, transport substrate = phosphatidylcholine; Abcg5 and Abcg8, transport substrate = CH), livers from noninfused Bsep−/− and Bsep+/+ mice (n = 6/group) were used. Plasma CH concentrations were also determined in noninfused Bsep−/− and Bsep+/+ mice (n = 7–10/group). Mice were anesthetized under isofurane anesthesia, after which they were terminated by cardiac puncture. Livers were excised, weighed, snap-frozen in liquid nitrogen, and stored at −80°C until further analysis.

Biochemical analytical procedures. Total BS concentrations were measured enzymatically as described (28). A commercially available kit was used for the determination of PL (Wako Chemicals, Neuss, Germany). Biliary CH was determined enzymatically (10) on a Novostar analyzer (BMG Labtech, Offenburg, Germany). Biliary BS composition was determined by capillary gas chromatography as described previously (22) after extraction of the BS from bile by use of Sep-Pack C-18 cartridges (Waters Associates, Milford, MA). Plasma CH concentrations were determined following the standard procedure at the Clinical Chemical Laboratory of the University Medical Centre Groningen, The Netherlands.

RNA isolation and RT-PCR. Total RNA was extracted from frozen tissues using TriReagent (Sigma, St. Louis, MO) and quantified spectrophotometrically. Single-stranded cDNA was synthesized according to Bloks et al. (5). Quantitative real-time PCR was performed using a 7900HT Fast Real-Time PCR system (Applied Biosystems, Foster City, CA), according to the manufacturer’s instructions. Primers and probes (Invitrogen, Breda, The Netherlands) were described previously (34). Membrane purification was assessed by determining expression of Na+-K+-ATPase and alkaline phosphatase activities in plasma membrane fractions compared with those in homogenates (23). The purities of the plasma membrane fractions isolated from Bsep−/− and Bsep+/+ control mice were similar. Plasma membranes (10 μg protein), normalized for enrichment in Na+-K+-ATPase and alkaline phosphatase, were electrophoresed through SDS-polyacrylamide gels (10%) and subsequently transferred to a nitrocellulose filter (GE Healthcare UK, Little Chalfont, UK). Ponceau S staining was performed to check for equal protein transfer. Blots were incubated with primary antibodies overnight at 4°C (23). Used antibodies were against Mdr3 (Santa Cruz Biotechnology, Santa Cruz, CA), Ntcp (40) and Mrp2 (27) (both were generous gifts from Dr. B. Stieger, University Hospital, Zurich, Switzerland), Bsep (47), and Abcg5 (gift from Dr. A. K. Groen, formerly employed at Academic Medical Center, Amsterdam, The Netherlands) (24). Immune complexes were detected using horseradish peroxidase (HRP)-conjugated antibodies (goat anti-mouse IgG2a-HRP from Southern Biotechnology Associates, Birmingham, UK, and donkey anti-rabbit IgG-HRP from GE Healthcare) and the SuperSignal West Pico Chemiluminescent Substrate (Pierce/Thermo Fisher Scientific, Rockford, IL). Protein band densitometry was performed by ChemiDoc XRS (Bio-Rad, Hercules, CA).

Statistics. Statistical analyses were performed using SPSS 12.0.2 for Windows (SPSS, Chicago, IL). All values are expressed as means ± SD. Differences between genotypes were evaluated using the Mann-Whitney U-test or, for the infusion experiments (Fig. 1), the ANOVA repeated measurements. A P value <0.05 was considered statistically significant.

RESULTS

Body weight and liver weight in Bsep−/− and Bsep+/+ mice. Table 1 lists the basal parameters obtained from Bsep−/− and control (Bsep+/+) mice. The body weight of Bsep−/− and Bsep+/+ mice was similar, whereas liver weight was higher in the former. Therefore, liver weight-to-body weight ratio was significantly higher in Bsep−/− mice.

Bile flow, biliary BS, and lipid secretion in Bsep−/− and Bsep+/+ mice. Following cannulation of the gallbladder, bile was collected for 90 min in 30-min fractions. During the first 90 min, bile flow and biliary secretion of BS, PL, and CH were relatively constant (Fig. 1, A and B, respectively). Under these basal conditions, bile flow did not differ significantly in Bsep−/− mice compared with Bsep+/+ mice (Table 1). Whereas biliary BS and PL excretion was unchanged, biliary CH excretion was about fivefold increased in Bsep−/− compared with Bsep+/+ mice (P < 0.001). The degree of “coupling” of biliary PL and CH to BS secretion can be expressed as the PL-to-BS and CH-to-BS molar ratio, respectively. Both the PL-to-BS and CH-to-BS molar ratios were significantly two- to fivefold increased in Bsep−/− mice during the course of the experiment. Because the molar ratios are independent of the liver weights, the difference in liver weights between the genotypes is not likely responsible for the higher lipids to BS ratio. In accordance with this assumption, the PL-to-BS and CH-to-BS ratios were not related to liver weights of Bsep−/− and Bsep+/+ mice (data not shown).

Effects of tauro-β-muricholate infusion on bile flow, biliary BS, and lipid secretion in Bsep−/− and Bsep+/+ mice. We infused TβMCA in stepwise increasing dosages and determined its effects on biliary excretion rates of BS, PL, and CH. During TβMCA infusion, bile flow in Bsep−/− mice did not increase, in contrast to that in Bsep+/+ (interaction genotype-time P < 0.0001; Fig. 1A). Biliary BS secretion in Bsep−/− mice was significantly lower during infusion (interaction genotype-time P < 0.0001; Fig. 1B), whereas biliary PL secretion increased similarly over time in both genotypes (P < 0.001; Fig. 1C). During TβMCA infusion, biliary CH secretion was significantly higher in Bsep−/−, but the difference disappeared at the highest infusion rates (Fig. 1D).
Relation between biliary BS and lipid secretion during TβMCA infusion. To compare BS-induced biliary lipid secretion in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice, biliary PL and biliary CH secretions were related to biliary BS secretion (Fig. 2). Both in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice, the previously described hyperbolic relations between biliary BS secretion and that of either PL or CH were observed. Profoundly more PL and CH were secreted at every level of BS secretion in Bsep<sup>−/−</sup> mice compared with control mice.

Table 1. Mouse characteristics, biliary secretion rates, and concentrations of major bile components under basal conditions in Bsep<sup>+/+</sup> and Bsep<sup>−/−</sup> mice

<table>
<thead>
<tr>
<th></th>
<th>Bsep&lt;sup&gt;+/+&lt;/sup&gt;</th>
<th>Bsep&lt;sup&gt;−/−&lt;/sup&gt;</th>
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<tbody>
<tr>
<td>Body wt, g</td>
<td>28.3 (25.1–37.8)</td>
<td>27.2 (20.2–33.9)</td>
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<tr>
<td>Liver wt, g</td>
<td>1.5 (1.0–1.7)</td>
<td>2.4 (1.1–2.9)**</td>
</tr>
<tr>
<td>Ratio liver wt/body wt</td>
<td>4.1 (3.8–6.6)</td>
<td>8.5 (4.6–10.8)**</td>
</tr>
<tr>
<td>Bile flow, µl·min&lt;sup&gt;−1&lt;/sup&gt;·100 g body wt&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>8.9 (6.5–12.2)</td>
<td>9.6 (4.2–19.0)</td>
</tr>
<tr>
<td>Bile salts secretion, nmol·min&lt;sup&gt;−1&lt;/sup&gt;·100 g body wt&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>389.8 (100.3–1506.6)</td>
<td>306.5 (96.3–1249.9)</td>
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<tr>
<td>Phospholipid secretion, nmol·min&lt;sup&gt;−1&lt;/sup&gt;·100 g body wt&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>32.4 (18.0–70.5)</td>
<td>50.9 (22.7–128.4)</td>
</tr>
<tr>
<td>Cholesterol secretion, nmol·min&lt;sup&gt;−1&lt;/sup&gt;·100 g body wt&lt;sup&gt;−1&lt;/sup&gt;</td>
<td>3.9 (2.4–9.7)</td>
<td>15.1 (7.4–28.7)**</td>
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<tr>
<td>Biliary bile salt concentration, mmol/l</td>
<td>49.7 (12.0–134.8)</td>
<td>32.6 (14.0–72.3)</td>
</tr>
<tr>
<td>Biliary phospholipid concentration, mmol/l</td>
<td>3.7 (2.0–9.6)</td>
<td>5.3 (1.4–13.4)</td>
</tr>
<tr>
<td>Biliary cholesterol concentration, mmol/l</td>
<td>0.4 (0.3–1.3)</td>
<td>1.9 (0.6–4.6)**</td>
</tr>
<tr>
<td>Phospholipids-to-bile salts molar ratio</td>
<td>0.09 (0.03–0.17)</td>
<td>0.17 (0.02–0.34)*</td>
</tr>
<tr>
<td>Cholesterol-to-bile salts molar ratio</td>
<td>0.01 (0.01–0.03)</td>
<td>0.05 (0.03–0.12)**</td>
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Data are averages of the first two fractions. Data are expressed as median values (range); n = 7–10 mice/group. Following gallbladder canulation, bile was collected in 30-min fractions for a total of 90 min. *P < 0.05 and **P < 0.01.

BS-dependent and -independent bile flow in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice. To assess whether a lower BSIF in Bsep<sup>−/−</sup> mice could explain the increased ratio of biliary lipid to biliary BS secretion, we estimated the latter in a BS secretion-flow graph (Fig. 3). Extrapolation of this relation to zero BS output gives the (theoretical) BSIF (y-axis intercept) (32, 46). BSIF was higher in Bsep<sup>−/−</sup> compared with Bsep<sup>+/+</sup> mice (11.8 vs. 7.9 µl·min<sup>−1</sup>·100 g body wt<sup>−1</sup>, respectively), indicating that the increased ratio (coupling)
Biliary BS concentration (111 mM) and composition (95–99% at the highest T) of lipid-to-BS secretion could not be attributed to a lower BSIF in Bsep<sup>−/−</sup> mice. Biliary BS composition in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice. To investigate whether the increase in biliary lipid secretion in Bsep<sup>−/−</sup> mice could be caused by a change in the biliary BS composition during TβMCA infusion, biliary BS composition was analyzed under basal conditions and following TβMCA infusion (Fig. 4). Under basal conditions, biliary BS of Bsep<sup>−/−</sup> mice were predominantly composed of β-muricholic acid (81% of total) in contrast to predominantly cholic acid in Bsep<sup>+/+</sup> mice (53% of total). At the end of the experiment, i.e., at the highest TβMCA infusion dosage (600 nmol/min), biliary BS concentration (~111 mM) and composition (~95–99% β-muricholate) were similar in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice. Despite this similarity, the ratio of either PL or CH to BS was still approximately twofold higher in Bsep<sup>−/−</sup> mice compared with Bsep<sup>+/+</sup> mice (Fig. 2). BS, PL, and CH transporter expression in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice. To investigate if increased BS-induced biliary lipid, PL, and CH secretion in Bsep<sup>−/−</sup> mice could be due to increased canalicular lipid transporter expression, mRNA and protein expression of relevant canalicular transport proteins in livers from noninfused Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice was determined. We did not attempt to determine mRNA or protein levels of relevant canalicular transport proteins in livers obtained after the infusion experiments because of the prolonged anesthesia (>4 h) and the possibility that mice were developing cholestasis at the end of the infusion period. As expected, Bsep mRNA was not detectable in the liver of (noninfused) Bsep<sup>−/−</sup> mice (Fig. 5). Hepatic mRNA expressions of Mdr2, Abcg5, and Abcg8 tended to be higher in Bsep<sup>−/−</sup> compared with Bsep<sup>+/+</sup> mice (45–55%, for Abcg5; P < 0.05). Western blotting was performed after isolation of plasma membranes. Western blotting of plasma membranes, normalized for enrichment in Na<sup>+</sup>-K<sup>+</sup>-ATPase and alkaline phosphatase, showed increased hepatic protein expressions of transport proteins Mdr2 and Abcg5 involved in canalicular PL and CH secretion but similar expression of the basolateral Ntcp transport protein (Fig. 6). Bsep protein could not be detected in livers of Bsep<sup>−/−</sup> mice in contrast to livers of Bsep<sup>+/+</sup> mice (Fig. 6).

Finally, we assessed whether the expression levels of relevant transcription factors were affected in Bsep<sup>−/−</sup> mice. Neither the mRNA levels of Fxr, Lxrα, or Lxrβ were significantly different in Bsep<sup>−/−</sup> mice compared with Bsep<sup>+/+</sup> mice (Bsep<sup>−/−</sup> vs. Bsep<sup>+/+</sup> mice: Fxr/18S, 0.83 ± 0.35 vs. 1.00 ± 0.20; Lxrα/18S, 1.23 ± 0.47 vs. 1.00 ± 0.10; Lxrβ, 1.12 ± 0.50 vs. 1.00 ± 0.29; all not significant).

**DISCUSSION**

BS stimulate biliary secretion of biliary lipids (PL and CH). In Bsep<sup>−/−</sup> mice, biliary secretion of various BS is decreased, but that of PL and CH is unexpectedly elevated (50). Our data indicate that the mechanism of increased biliary lipid secretion in Bsep<sup>−/−</sup> mice can be attributed to elevated hepatic expression of canalicular lipid transport proteins and not to changes in BS composition or BSIF.
In Bsep<sup>−/−</sup> mice, biliary bile flow and biliary BS secretion showed a relatively large variation under basal unstimulated conditions. The biliary BS composition was different among the genotypes. Biliary BS of Bsep<sup>−/−</sup> mice consisted predominantly of \( \alpha \)-muricholic acid (81% of total), with a small amount of cholic acid (6% of total), whereas that of control mice was predominantly composed of cholic acid (53% of total) and to a lesser extent of \( \beta \)-muricholic acid (26% of total; Fig. 4). The coupling of biliary PL or CH secretion to that of BS was higher in Bsep<sup>−/−</sup> mice, both under basal unstimulated conditions and during TβMCA infusion. A higher biliary lipid secretion was also obtained under nonstimulated conditions by Wang et al. in Bsep<sup>−/−</sup> mice inbred to a C57BL/6J background for three to four generations (49, 50).

The amount of PL molecules that is secreted per BS molecule into bile, i.e., the degree of coupling or the ratio between PL and BS secretion, is regulated by at least six factors (42): 1) intracanalicular BS concentration; 2) BS composition (hydrophobicity) (7, 17, 18); 3) magnitude of BSIF (46); 4) biliary concentration of uncoupling organic anions, e.g., bilirubin ditaurate, ampicillin, or sulfobromophthalein (1, 2, 20, 29, 43, 51); 5) expression level of the canalicular PL transporter Mdr2; and 6) the lipid composition of the bile canalicular membrane (52). The biliary secretion of CH is strongly dependent on the expression levels of the transport proteins Abcg5 and Abcg8 (4). To address the mechanism of the increased ratio of biliary lipids to biliary BS, we herewith review the six factors demonstrated earlier to affect the biliary PL-to-BS ratio (1, 2, 7, 17, 18, 20, 29, 45, 46, 51, 52). Secretion of PL and CH usually correlates positively with changes in the amount of BS secreted in vivo until a certain plateau is reached (21, 43, 45). The differences in the hyperbolic relations between biliary BS and lipid secretion (Fig. 2) clearly indicate that increased BS-induced biliary lipid secretion in Bsep<sup>−/−</sup> mice is not due to biliary BS concentration. The ratio of biliary lipid to BS secretion was two- to threefold higher in Bsep<sup>−/−</sup> mice, implying that during the TβMCA infusion each BS molecule was associated with more PL and CH molecules. Our data also excluded that differences in BS composition were responsible for the observed increased biliary lipid to BS coupling in Bsep<sup>−/−</sup> mice: at the end of the infusion period, TβMCA was the predominant BS in both genotypes, but, nevertheless, the lipid-to-BS ratio was higher in Bsep<sup>−/−</sup> mice (Fig. 2). Infusion of BS into rats above a certain dosage results in a decline in biliary PL and CH secretion (3, 16, 52, 53). Based on the observation that PL secretion was the highest at the final

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**Fig. 4.** Biliary bile salt composition in Bsep<sup>−/−</sup> and Bsep<sup>+/+</sup> mice under basal conditions (A) and after TβMCA infusion (B). \( \alpha \)-M, \( \alpha \)-muricholate; DC, deoxycholate; C, cholate; CDC, chenodeoxycholate; HDC, hexyloxydeoxycholate; UDC, ursodeoxycholate; \( \beta \)-M, \( \beta \)-muricholate; \( \Delta 22 \)-M, \( \Delta 22 \)-muricholate; \( \omega \)-M, \( \omega \)-muricholate. Data are expressed as means ± SD, n = 7–10/group, \(* P < 0.05\) and \(** P < 0.01\).
The increased mRNA and protein expression of Abcg5 in canalicular membrane could be a consequence of (hydrophobic) BS accumulation in the liver of Bsep−/− mice. Bsep inactivation changes biliary BS composition, least effecting the secretion of the hydrophilic muricholic acid. It has been demonstrated that hydrophobic BS can induce hepatic mRNA expression of Mdr2 in mice via the nuclear receptor Fxr (8, 13). Thus it could be that accumulated intrahepatocytic BS, directly or indirectly, induce the expression of canalicular transporters in Bsep−/− mice via Fxr or other nuclear receptors (12, 36, 37). The steady-state mRNA levels of the nuclear transcription factors Fxr, Lxrα, and Lxrβ did not differ significantly between Bsep−/− and Bsep+/+ mice, but this does not exclude that the (downstream) signaling of the transcription factors is affected by intracellularly accumulated BS. Taken together, our data show that the increased biliary lipid secretion in Bsep−/− mice is still BS dependent and corresponds with elevated hepatic expression of transport proteins involved in canalicular lipid secretion. Likewise, it is possible that the accumulation of BS in PFIC2 patients also affects the expression of relevant canalicular transport proteins, and thereby contributes to the biliary phenotype of the disease. However, this remains to be investigated.

An interesting observation from the present studies concerns the apparent lower BS-dependent flow in Bsep−/− mice com-

Fig. 5. Hepatic mRNA expression levels of genes encoding for canalicular transport proteins for bile salts (Bsep), phospholipids (Mdr2), and cholesterol (Abcg5/Abcg8) normalized to 18S. Hepatic samples were obtained from noninfused Bsep−/− and Bsep+/+ mice. Data are expressed as means ± SD, n = 6/group, *P < 0.05.

Fig. 6. Western blot of plasma membrane fractions isolated from Bsep−/− and Bsep+/+ mice. Shown are duplicate lanes with plasma membrane fractions pooled from 3 mice. Analysis was done as described in MATERIALS AND METHODS. In short, equal amounts of plasma membranes (10 μg protein) were loaded, normalized for enrichment in Na⁺/K⁺-ATPase and alkaline phosphatase. The purities of the plasma membrane fractions isolated from Bsep−/− and Bsep+/+ control mice were similar. Based on densitometry, canalicular protein expression of Mdr2, Abcg5, and Ntcp in Bsep−/− mice was 1.6-fold higher in Bsep−/− mice compared with Bsep+/+ mice, whereas expression of Bsep could only be detected in Bsep+/+ mice, and expression of Ntcp in Bsep−/− mice was 0.8-fold of that in Bsep+/+ mice (for details please see MATERIALS AND METHODS).
pared with Bsep<sup>+/+</sup>: the slope of the bile secretion-flow correlation is more shallow in the former. The underlying mechanism of this phenomenon is unclear. It is tempting to speculate that the increased biliary PL and CH secretion in Bsep<sup>−/−</sup> mice aggregates into larger mixed micelles in the canaliculus, which decreases the choleretic potential of the BS. Previously, we demonstrated in rats with a chronic external bile fistula that the duodenal administration of PL and CH could partly reconstitute fat absorption (30). The present observation of increased coupling of biliary lipids in Bsep<sup>−/−</sup> mice poses the question whether the increased coupling preserves the fat absorption in Bsep<sup>−/−</sup> mice and contributes to the relative benign phenotype in Bsep<sup>−/−</sup> mice compared with PFIC2 patients.

In conclusion, the mechanism of increased biliary lipid secretion relative to that of BS in Bsep<sup>−/−</sup> mice corresponds with an increased expression of the responsible canalicular transporter proteins.

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DISCLOSURES

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

AUTHOR CONTRIBUTIONS


REFERENCES


