Chapter 5

Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice: efficient intestinal bile salt absorption in the absence of ileal bile acid-binding protein

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ABSTRACT

Background/Aims: The bile salt-activated farnesoid X receptor (FXR; NR1H4) controls expression of several genes considered crucial in maintenance of bile salt homeostasis. We evaluated the physiological consequences of FXR deficiency on bile formation and on the kinetics of the enterohepatic circulation of cholate, the major bile salt species in mice.

Methods: The pool size, fractional turnover rate, synthesis rate, and intestinal absorption of cholate were determined by stable isotope dilution and were related to expression of relevant transporters in the liver and intestines of FXR-deficient (Fxrand(-/)) mice.

Results: Fxrand(-/)) mice showed only mildly elevated plasma bile salt concentrations associated with a 2.4-fold higher biliary bile salt output, whereas hepatic mRNA levels of the bile salt export pump (Bsep) were decreased. Cholate pool size and total bile salt pool size were increased by 67% and 39%, respectively, in Fxrand(-/)) mice compared to wild-type mice. The cholate synthesis rate was increased by 85% in Fxrand(-/)) mice, coinciding with a 2.5-fold increase in cholesterol 7α-hydroxylase (Cyp7a1) and unchanged sterol 12α-hydroxylase (Cyp8b1) expression in the liver. Despite a complete absence of ileal bile acid-binding protein (Ibapb) mRNA and protein, the fractional turnover rate and cycling time of the cholate pool were not affected. The calculated amount of cholate reabsorbed from the intestine per day was ~2-fold higher in Fxrand(-/)) than in wild-type mice.

Conclusions: Absence of FXR in mice is associated with defective feedback inhibition of hepatic cholate synthesis, which leads to enlargement of the circulating cholate pool with an unaltered fractional turnover rate. The absence of Ibapb does not negatively interfere with the enterohepatic circulation of cholate in mice.
INTRODUCTION

Bile salts, synthesized from cholesterol in the liver, have a number of important physiological functions in the body. Bile salts are essential for generation of bile flow and for biliary excretion of cholesterol. In the intestine, they are required for efficient absorption of dietary fat and fat-soluble vitamins. Finally, there is a growing body of evidence that bile salts are involved in the control of high density lipoprotein (HDL) and very low density lipoprotein (VLDL) metabolism. To ensure the presence of adequate concentrations of these biologically active compounds at the sites of their actions, i.e., liver, biliary tract and intestine, bile salts are maintained within the enterohepatic circulation by the combined actions of transporter systems in the liver and intestine. The bile salt export pump (Bsep; Abcb11) has been identified as the major canalicular bile salt-transporting protein. Intestinal absorption of bile salts is mediated, to a large extent, by the apical sodium-dependent bile salt transporter (Asbt; Slc10a2) localized in the terminal ileum. The Asbt splice variant t-Asbt is localized basolaterally and may be involved in efflux of bile salts from enterocytes toward portal blood. Ileal bile acid-binding protein (Ibabp) is a small soluble protein of which expression is restricted to the terminal ileum. Ibabp is thought to be involved in facilitating uptake of bile salts and their intracellular trafficking in the small intestine. After their reabsorption, bile salts are taken up by hepatocytes from portal blood via the Na+-taurocholate co-transporting polypeptide (Ntcp; Slc10a1) and Na+-independent organic anion-transporting polypeptides, including Oatp1 (or Slc21a1). Only a relatively small fraction of bile salts escapes intestinal absorption and is lost into the feces, which is compensated for by de novo bile salt biosynthesis in the liver. Therefore, under steady state conditions, bile salt pool size remains constant.

Recently, it has been become clear that bile salts exert regulatory actions on expression of specific genes via the nuclear farnesoid X receptor (FXR; NR1H4). Bile salts such as chenodeoxycholate, deoxycholate, cholate, and their conjugates are natural ligands for FXR. Activated FXR inhibits expression of the gene encoding cholesterol 7α-hydroxylase (Cyp7a1), which catalyzes the first and rate-controlling step of bile salt synthesis. This repression is achieved indirectly via a coordinated regulatory cascade involving FXR-mediated induction of the small heterodimer partner (SHP; NROB2), which, in turn, inhibits the activity of the tissue-specific factor liver receptor homologue-1 (LRH-1; NR5A2), which controls expression of Cyp7a1. In this way, bile salts exert negative feedback control on their own synthesis. Sterol 12α-hydroxylase (Cyp8b1), the enzyme that controls the ratio in which the primary bile salt species cholate and chenodeoxycholate are formed, seems to be under the negative control of bile salts in an FXR-dependent manner as well. Activated FXR also controls expression of hepatic bile salt transporters, i.e., it induces the expression of Bsep and down-regulates the expression of Ntcp via SHP. A recent study showed that mouse (but not rat) Asbt is also subjected to negative feedback regulation mediated by FXR via SHP-dependent repression of LRH-1 activation. Finally, bile salts strongly induce the expression of intestinal Ibabp in an FXR-dependent manner.
Therefore, in general terms, FXR appears to control various crucial steps in bile salt metabolism, which may provide possibilities for therapeutic interventions, e.g., aimed at treatment of hypercholesterolemia or of cholestatic liver diseases. However, in view of the wide variety of genes that are controlled by FXR, it is essential to understand the impact of induced alterations in FXR activity, not only on expression of individual genes, but also on metabolism at the whole body level. Recent studies in chow-fed FXR-deficient mice by Sinal et al. showed increased hepatic Cyp7a1 mRNA levels, but, very surprisingly, reductions in total bile salt pool size and fecal bile salt loss. Counterintuitively, these data imply that, in the absence of functional FXR, bile salt synthesis would actually be suppressed. Other mouse models with increased Cyp7a1 expression showed, as expected, increased bile salt synthesis rates and expansion of bile salt pool sizes. This may imply that FXR deficiency has an impact on maintenance of bile salt pool size at other levels, for instance in the intestine. To address this issue further, we have evaluated parameters of the enterohepatic circulation of cholate, quantitatively the major bile salt species in the mouse, in relation to bile formation and the expression of transport proteins in a new mouse model of FXR deficiency generated by the ‘classical’ homologous recombination approach. For quantitation of cholate kinetic parameters, a recently developed stable isotope dilution technique was used.

This study demonstrates that, in accordance with derepressed transcription of Cyp7a1, Fxr(-/-) mice do show an increased cholate synthesis rate. Interestingly, the calculated intestinal cholate reabsorption was markedly increased despite a complete absence of Ibabp mRNA and protein, leading to an enlarged bile salt pool size. This finding may imply that Ibabp functions as a negative regulator rather than as a positive regulator of intestinal bile salt reabsorption in the mouse.

EXPERIMENTAL PROCEDURES

Animals
Fxr(-/-) mice were generated by Deltagen, Inc. (Redwood City, CA) using standard gene-targeting methods. To disrupt the Fxr locus, a 292 bp fragment corresponding to a segment of exon 2 was replaced by a phosphoglycerate kinase promoter-driven neomycin resistance cassette in a targeting vector. The construct was linearized and electroporated into embryonic stem cells derived from the 129/OlaHsd strain. Cells harboring the desired mutation were identified by positive selection and injected into recipient C57BL/6J blastocysts to produce chimeras, which were used for the generation of F1 heterozygotes. F2 wild-type, heterozygous, and homozygous mice were produced from F1 intercross in the expected mendelian ratios. Genotyping was accomplished by PCR using primer pairs specific for the wild-type Fxr allele (5’-GTT GTA GTG GTA CCC AGA GGC CCT G-3’ and 5’-TAT GCT AAC AGA ACA CGC GGC AGG C-3’) or the mutant allele (5’-GTT GTA GTG GTA CCC AGA GGC CCT G-3’ and 5’-GGG TGG GAT TAG ATA AAT GCC TGC TCT-3’).
Male homozygous (Fxr(-/-)), heterozygous (Fxr(+/-)) and wild-type (Fxr(+/-)) mice (C57BL/6Jx129/OlaHsd) of 25-30 g were bred at the animal facility of the
University of Groningen and used for these studies. Mice were housed in a light- and temperature-controlled facility. Food and water were available *ad libitum*, and mice were maintained on standard laboratory chow (RMH-B; Hope Farms BV, Woerden, The Netherlands). All experiments were approved by the Ethical Committee on animal testing of the University of Groningen.

**Materials**

[2,2,4,4-2H₄]-cholate ([2H₄]-cholate, isotopic purity 98%) was obtained from Isotec (Miamisburg, OH). Cholylglycine hydrolase from *Clostridium perfringens* (welchii) was purchased from Sigma Chemicals (St. Louis, MO). Pentafluorobenzylbromide (PFB) was purchased from Fluka Chemie (Buchs, Neu-Ulm, Switzerland). All other chemicals and solvents used were of the highest purity commercially available.

**Methods**

Mice were anesthetized with a mixture of Hypnorm (1ml/kg) and Diazepam (10 mg/kg) (Janssen Pharmaceutica, Beerse, Belgium). Six *Fxr*(-/-), six *Fxr*(+/-), and six wild-type mice were subjected to bile duct cannulation for collection of bile. During the 30 min bile collection period, animals were placed in a humidified incubator to ensure maintenance of body temperature. Bile flow was determined gravimetrically, assuming a density of 1 g/ml for bile. Bile was stored at -20°C until analysis. Blood was obtained by cardiac puncture and collected in EDTA-containing tubes. Plasma was obtained by centrifugation at 9000 rpm for 10 min and stored at -80°C until analyzed. The livers were excised, weighed, cut into small pieces, snap-frozen in liquid nitrogen, and stored at -80°C until used for isolation of RNA and for biochemical analyses. Samples for microscopic evaluation were frozen in isopentane and stored at -80°C or fixed in paraformaldehyde for hematoxylin/eosin and oil red O staining. The small intestine was rinsed with phosphate-buffered saline (PBS) containing phenyl-methyl-sulfonylfluoride (PMSF) (Roche Applied Science) to prevent protein degradation and divided in proximal, mid and distal parts. Tissue samples were immediately frozen in liquid nitrogen and stored at -80°C for membrane preparation and for RNA isolation. Feces were lyophilized, weighed, and homogenized. To collect urine, four wild-type and four *Fxr*(-/-) mice were placed in metabolic cages that allowed for separate collection of feces and urine for a period of 24 h.

In a second experiment, 240 µg of [3H₄]-cholate in a solution of 0.5% NaHCO₃ in PBS (pH = 7.4) was intravenously administered to male *Fxr*(+/+) and *Fxr*(-/-) mice. Subsequently, blood samples (100 µL) were obtained at 24, 36, 48, and 60 hours after administration of [3H₄]-cholate. Plasma was obtained by centrifugation at 9000 rpm for 10 min and stored at −20°C until analyzed. After 60 h, the mice were anesthetized with Hypnorm and Diazepam (see above) and subjected to bile duct cannulation. To ensure that hepatic production was accurately measured, bile produced during the initial 5 min after cannulation was discarded, and bile was sampled for 30 min thereafter.

**Steady-state mRNA levels determined by real-time quantitative PCR**

Total RNA was isolated from frozen mouse liver and intestinal tissue using TRIzol Reagent (Invitrogen) according to the manufacturer’s instructions. RNA
was checked on an agarose gel for integrity, and RNA concentration was measured using the Ribogreen RNA quantitation kit (Molecular Probes, Leiden, The Netherlands). Reverse transcription was performed on 2.5 mg of total RNA using random primers in a final volume of 38 ml (Reverse Transcription System, Promega, Madison, WI) for 10 min at 25°C, followed by one hour at 45°C. Samples were subsequently heated for 5 min at 95°C to terminate the reverse transcription reaction. Real-time quantitative PCR was performed on cDNA samples as described by Heid et al. to quantify mRNA levels. Primer and probe sequences for β-actin, Fxr (Nr1h4), Asbt (Slc10a2), truncated Asbt (t-Asbt), and Ibabp have been described by Hulzebos et al. Primer and probe sequences for Abcg5, Abcg8, Bsep (Abcb11), Cyp7a1, Cyp27, Mdr2 (Abcb4), Ntcp (Slc10a1), and Oatp1(Slc21a1) have been described by Plösch et al. The following primer sequences were used for Mrp2 (Abcc2): sense primer GGA TGG TGA CTG TGG GCT GAT, anti-sense primer GGC TGT TCT CCC TTC TCA TGG and probe AGC TGC ATC GTC GAT AAT TTC TCA TGG and probe AGC TGC ATC GTC GAT AAT TTC TCA CAC A (Accession number NM_013806). For Cyp8b1; sense primer AAG GGT GTC TTC CTG AGC TT, anti-sense primer AAC AGC TCA TCG GCC TCA TC and probe CGG CTA CAC CAA GGA CAA GCA GCA AG (Accession number NM_010012). For Shp (Nr0b2); sense primer AAG GGC ACG ATC CTC TTC AA, anti-sense primer CTG TTG CAG GTG TGC GAT GT and probe ATG TGC CAG GCC TCC GTG CC (Accession number L76567). For Fic1 (Atp8b1); sense primer CAC ACC AGG ATG GAG AAT CAG A, anti-sense primer GCC AGG AGC CAG TGA TTA and probe TCT CTG CAG AAT TTG CAC CTC CTG TG (Accession number AF395823). And for Mrp3 (Abcc3); sense primer TCC CAC TTT TCG GAG ACA GTA AC, anti-sense primer ACT GAG GAC CTT GAA GTC TTG GA and probe CAC CAG TGT CAT TCG GGC CTA TGG C (Accession number B5F84533). Primers and detection probes for the gene of interest, labeled with a fluorescent reporter dye (6-carboxyfluorescein) and a fluorescent quenching dye (6-carboxytetramethylrhodamine), were added. Fluorescence was measured by an ABI Prism 7700 Sequence Detector version 1.6 software (Perkin Elmer Life Sciences, Foster City, CA). For every PCR reaction, β-actin was used as the internal control. The cycle number at the threshold (CT), after which the intensity of reporter fluorescent emission increases, was used to quantitate the PCR product.

**Preparation of intestinal membranes for protein analysis**

Intestinal brush border membranes were isolated as described by Schmitz et al. Total protein concentration of intestinal homogenates and brush-border membrane fractions was determined using the method described by Lowry et al.

**Western blotting**

Approximately 2.5 µg of protein of homogenates and of intestinal brush-border membrane fraction of each group was separated using 4-15% Tris-HCL ready gradient gels (Bio-Rad Laboratories, Hercules, CA) and transferred to nitrocellulose (Amersham Biosciences, Buckinghamshire, UK) using a tankblotting system (Bio-Rad laboratories). The Ibabp protein content of intestinal homogenates and the Asbt protein content of brush-border membranes were determined.
using recombinant anti-murine Ibabp antibody\(^\text{28}\) and polyclonal anti-hamster Asbt antibody\(^\text{29}\), respectively. The blots were incubated with the first antibody diluted in Tris-buffered saline (TBS) containing 5% dried milk powder and 0.1% polyoxyethylene sorbitan monolaurate (Tween 20; Sigma), washed in TBS / 0.1% Tween 20 and incubated with horseradish peroxidase-labeled donkey anti-rabbit IgG (dilution 1:1000; Amersham Biosciences). Detection was done using the ECL Western blot kit (Amersham Biosciences).

**Analyses**

Bile salt concentrations in plasma, bile, feces and urine were determined by an enzymatic fluorimetic assay\(^\text{30}\). Levels of biliary cholesterol and phospholipids were measured as described by Kuipers \textit{et al.}\(^\text{31}\). Aspartate transaminase (ASAT) and alanine transaminase (ALAT) activities and total bilirubin concentrations in plasma were determined by routine clinical chemical procedures.

**Gas chromatography**

Bile salt composition of bile samples was determined by capillary gas chromatography on a Hewlett-Packard gas chromatograph (HP 5880A) equipped with a 50 m x 0.32 mm CP-Sil-19 fused silica column (Chrompack BV, Middelburg, The Netherlands). For this purpose, bile salts were converted to their methyl ester/trimethylsilyl derivatives\(^\text{21}\).

**Thin-layer chromatography**

Conjugation patterns of biliary bile salts were analyzed by thin-layer chromatography (TLC) on precoated silica gels (60F254, Merck, Darmstadt, Germany) using n-butanol-acetic acid-water (10:2:1) as solvent system\(^\text{32}\).

**Gas-liquid chromatography/electron capture negative chemical ionization mass spectrometry**

Plasma samples were prepared for isotopic analysis of bile salts by gas chromatography mass spectrometry (GC-MS) as described\(^\text{21}\). All analyses were performed on a Finnigan SSQ7000 Quadrupole GC-MS instrument. Gas chromatographic separation was performed on a 15m x 0.25 mm column, 0.25-mm film thickness (AT-5MS, Alltech Associates Inc., Deerfield, IL).

**Isotope dilution technique calculations**

The isotope dilution technique has been described in detail by Hulzebos \textit{et al.}\(^\text{21}\). Enrichment was defined as the increase of M4-cholate/ M0-cholate relative to baseline measurements after administration of \(^{[2H_4]}\)-cholate and is expressed as the natural logarithm of atom percent excess (ln APE) value. The decay of ln APE in time was calculated by linear regression analysis for the individual mice. From the linear decay curve thus obtained, the fractional turnover rate (FTR) and pool size of cholate were calculated. The FTR (per day) equals the slope of the regression line. The pool size (mmol/100g) was determined according to the formula: \((D \cdot b \cdot 100) / e^a - D\), where ‘D’ is the administered amount of label, ‘b’ is the isotopic purity, and ‘a’ is the intercept on the y-axis of the ln APE versus time curve. The cholate synthesis rate (mmol/100g/day) was determined...
by multiplying pool size and FTR.

**Enterohepatic cycling time and intestinal reabsorption of cholate**

The cholate cycling time, *i.e.*, the time it takes the cholate pool to circulate a single time in the enterohepatic circulation, was calculated by dividing the cholate pool size (mmol/100g) by the biliary secretion rate of cholate (mmol/100g/h). The cholate biliary secretion rate was calculated by multiplying the bile flow (mL/100g/h) by the cholate concentration (mM) in a single 30 min fraction, obtained from 5 to 35 min after cannulation of the gallbladder. The amount of cholate reabsorbed per day was calculated by multiplying cholate pool size by cycling frequency and subsequent subtraction of the daily cholate synthesis rate.

**Statistical analyses**

All results are presented as means ± SD. Differences between the two or three groups were determined by t test or one-way analysis of variance (ANOVA), with post hoc comparison by Newman-Keuls t test, respectively. The level of significance for all statistical analyses was set at *p* < 0.05. Analyses were performed using SPSS for Windows software (SPSS, Chicago, IL).

![Figure 1. Targeted disruption of the murine *Fxr* gene. (A) Genomic organization of the wild-type allele and of the disrupted allele arising after homologous recombination. Neo′, neomycin resistance cassette; pA, polyadenylation signal. (B) PCR genotyping of wild-type (*Fxr*+/+), *Fxr*(+/−) and *Fxr*(−/−) mice. Genomic DNA was isolated from animals of the indicated genotype and amplified via PCR using allele-specific primers described under Experimental Procedures. Products were separated by agarose gel electrophoresis. (C) Northern blot analysis of hepatic RNA from wild-type(*+/+*) and *Fxr*(−/−) mice. Poly (A+) RNA was isolated from livers and pooled within genotype groups (*n*=5). Aliquots (3 µg) were separated by gel electrophoresis, transferred to nylon membranes, and hybridized to a radiolabeled FXR probe representing a 837-bp BamHI fragment containing the ligand-binding domain (upper panel). The filters were stripped and reprobed with a cDNA encoding rat cyclophilin (lower panel).]
RESULTS

Deletion of the Fxr gene
Mutation of the Fxr gene was accomplished by replacement of 292 bp from exon 2 with a neomycin resistance cassette conferring antibiotic resistance (Figure 1A). Homologous recombination in embryonic stem cells, injection into blastocysts, and transmission of the mutation through the mouse germ line were carried out by standard methods. The loss of ~97 amino acids encoded by exon 2 was anticipated to remove a large part of the DNA-binding domain of the FXR protein. PCR analysis (Figure 1B) of genomic DNA confirmed the predicted recombinations. RNA blotting revealed a Fxr transcript of ~2.0 kb in the livers of wild-type mice, whereas no transcript was detectable in Fxr(-/-) mice (Figure 1C).

Animal characteristics
The body and liver weights of Fxr(-/-) and Fxr(+/-) mice at three months of age were slightly higher than those of wild-type mice, but liver weight/body weight ratios were not affected (Table 1). There were no differences in plasma alanine transaminase (ASAT), aspartate transaminase (ALAT) and bilirubin concentrations between Fxr(-/-), Fxr(+/-) and Fxr(+/+) mice. Plasma bile salt concentrations were only slightly increased in Fxr(-/-) mice, in contrast to previously reported data in another strain of Fxr(-/-) mice described by Sinal et al.13. Accordingly, urinary bile salt loss was not significantly different between wild-type and Fxr(-/-) mice, i.e., 67.4 ± 14.6 nmol/day vs. 43.4 ± 11.0 nmol/day, respectively. Examination of hematoxylin- and eosin-stained liver sections of wild-type, Fxr(+/-) and Fxr(-/-) mice did not reveal any overt abnormalities in FXR-deficient mice (data not shown).

Table 1. Body and liver weights and plasma liver function parameters in wild-type, Fxr(+/-) and Fxr(-/-) mice

<table>
<thead>
<tr>
<th>Strain</th>
<th>wild-type</th>
<th>Fxr(+/-)</th>
<th>Fxr(-/-)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>23.0 ± 1.1</td>
<td>26.5 ± 1.7*</td>
<td>29.2 ± 3.1*</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>1.1 ± 0.1</td>
<td>1.4 ± 0.1*</td>
<td>1.5 ± 0.2*</td>
</tr>
<tr>
<td>Ratio LW/BW</td>
<td>0.049 ± 0.004</td>
<td>0.051 ± 0.006</td>
<td>0.053 ± 0.006</td>
</tr>
<tr>
<td>ASAT (U/L)</td>
<td>98 ± 18</td>
<td>83 ± 14</td>
<td>120 ± 64</td>
</tr>
<tr>
<td>ALAT (U/L)</td>
<td>40 ± 8</td>
<td>36 ± 12</td>
<td>63 ± 32</td>
</tr>
<tr>
<td>Bilirubin (mmol/L)</td>
<td>13.6 ± 2.6</td>
<td>13.0 ± 1.9</td>
<td>12.5 ± 1.0</td>
</tr>
<tr>
<td>Bile salts (mmol/L)</td>
<td>18.1 ± 3.6</td>
<td>21.6 ± 3.9</td>
<td>27.2 ± 7.4*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD (n = 6 per group). *Significant difference between wild-type and Fxr(+/-) or Fxr(-/-) mice. BW, body weight; LW, liver weight; ASAT, aspartate transaminase; ALAT, alanine transaminase.

Effects of FXR deficiency on bile formation and bile composition
FXR deficiency in mice was associated with an increase in bile flow (Table 2). The concentrations of phospholipids and cholesterol in bile did not differ among the three groups, but the biliary bile salt concentration was significantly increased in Fxr(-/-) mice. As a consequence, biliary output rates of bile salts were significantly increased in these animals. Output rates of phospholipid and cholesterol tended to
be increased in Fxr(−/−) mice, but differences did not reach statistical significance. Because biliary secretion of phospholipids and cholesterol is tightly coupled to that of bile salts, the output rates of these biliary lipids were also expressed relative to those of bile salts (Table 2). It is evident that, both for phospholipids and cholesterol, these ratios were lower in Fxr(−/−) mice than in wild-type mice. When
biliary bile salt output rates were plotted against bile flow for the individual mice of the three groups, the classical linear relationship between these parameters was observed (Figure 2). This strongly indicates that the bile formation process itself is not affected by FXR deficiency and that the higher bile flow rate in \( Fxr^{(-/-)} \) mice is caused by the higher bile salt output.

Analysis of biliary bile salt composition (Table 3) revealed that, in all three groups, cholate constituted the major fraction of biliary bile salts and that this fraction was higher in \( Fxr^{(-/-)} \) mice than in the other two groups. Accordingly, the relative contents of \( \alpha \)-muricholate and chenodeoxycholate were significantly decreased in \( Fxr^{(-/-)} \) mice. Thin-layer chromatography revealed that essentially all biliary cholate was conjugated to taurine in wild-type as well as in \( Fxr^{(-/-)} \) mice (data not shown). Despite the fact that bile salt-conjugated enzymes have recently been identified as FXR target genes\(^{34}\), unconjugated bile salts were undetectable by this procedure in bile of wild-type and \( Fxr^{(-/-)} \) mice.

**Table 3. Biliary bile salt composition (% total) in wild-type, \( Fxr^{(+/-)} \) and \( Fxr^{(-/-)} \) Mice**

<table>
<thead>
<tr>
<th>Strain</th>
<th>wild-type</th>
<th>( Fxr^{(+/-)} )</th>
<th>( Fxr^{(-/-)} )</th>
</tr>
</thead>
<tbody>
<tr>
<td>Deoxycholate</td>
<td>2.7 ± 1.1</td>
<td>3.2 ± 1.3</td>
<td>3.3 ± 1.8</td>
</tr>
<tr>
<td>( \alpha )-Muricholate</td>
<td>3.8 ± 0.4</td>
<td>3.0 ± 0.6*</td>
<td>1.7 ± 0.3*</td>
</tr>
<tr>
<td>( \beta )-Muricholate</td>
<td>16.0 ± 2.5</td>
<td>16.3 ± 2.5</td>
<td>11.8 ± 4.5</td>
</tr>
<tr>
<td>( \omega )-Muricholate</td>
<td>9.8 ± 2.2</td>
<td>9.1 ± 3.2</td>
<td>6.3 ± 3.4</td>
</tr>
<tr>
<td>HDC</td>
<td>3.4 ± 3.0</td>
<td>1.9 ± 1.9</td>
<td>0.6 ± 0.2</td>
</tr>
<tr>
<td>Chenodeoxycholate</td>
<td>2.7 ± 1.0</td>
<td>2.0 ± 0.3</td>
<td>1.4 ± 0.4*</td>
</tr>
<tr>
<td>Cholate</td>
<td>61.6 ± 4.7</td>
<td>64.6 ± 4.1</td>
<td>74.5 ± 7.3*</td>
</tr>
</tbody>
</table>

Values are expressed as means ± SD (n = 6 mice per group). *Significant difference between wild-type and \( Fxr^{(+/-)} \) or \( Fxr^{(-/-)} \) mice.

Steady-state mRNA levels of genes involved in bile salt synthesis and bile formation

Real-time quantitative PCR was used to evaluate hepatic expression of specific genes as influenced by FXR deficiency (Figure 3). As expected, expression of \( Shp \) tended to be lower in \( Fxr^{(-/-)} \) mice. \( Cyp7a1 \) was clearly increased in \( Fxr^{(-/-)} \) mice, whereas the expression levels of \( Cyp27 \) and \( Cyp8b1 \) were not significantly affected (Figure 3A). The mRNA levels of the gene encoding the canalicular bile salt transporter \( Bsep \), a well-known FXR target gene\(^{14,15}\), were significantly decreased in \( Fxr^{(-/-)} \) mice (Figure 3B). Expression of other transporters genes relevant to bile formation, such as the phospholipid translocator \( Mdr2 \), \( Ntcp \), and the putative cholesterol transporters \( Abcg5/g8 \), was not changed by FXR deficiency. Likewise, no effects on expression of \( Oatp1 \), \( Mrp2 \), and \( Mrp3 \) were observed.

Effects of FXR deficiency on kinetic parameters of cholate metabolism

To evaluate the physiological consequences of the observed changes in expression of bile salt synthesis and transporter genes, kinetic parameters of the enterohepatic circulation of cholate were determined by stable isotope dilution\(^{21}\). Because of the small differences between the heterozygotes and wild-type mice with respect to
Figure 3. Steady-state mRNA levels of genes involved in bile salt synthesis and transport in livers of wild-type, $Fxr^{(+/-)}$ and $Fxr^{(-/-)}$ mice. Total mRNA was isolated from the livers of wild-type (white bars), $Fxr^{(+/-)}$ (grey bars) and $Fxr^{(-/-)}$ (black bars) mice, transcribed into cDNA, and subjected to real-time PCR as described in Experimental Procedures. (A) Hepatic mRNA levels of $Fxr$, $Shp$, $Cyp7a1$, $Cyp27$ and $Cyp8b1$. (B) Hepatic mRNA levels of $Bsep$, $Mdr2$, $Abcg5$, $Abcg8$, $Ntcp$, $Oatp1$, $Mpr2$ and $Mpr3$. $n = 5$ for all groups. *Significant difference between wild-type and $Fxr^{(+/-)}$ or $Fxr^{(-/-)}$ mice.

Figure 4. Decay of intravenously administered $[^2H_4]$-cholate in wild-type and $Fxr^{(-/-)}$ mice. A dose of 240 µg of $[^2H_4]$-cholate was intravenously injected into wild-type (open symbols) and $Fxr^{(-/-)}$ (closed symbols) mice, and blood samples were collected at 24, 36, 48 and 60 h after injection for determination of plasma cholate enrichments by GC-MS as described under Experimental Procedures. Values are expressed in a logarithmic fashion, and the pool size ($y$-intercept), fractional turnover rate (slope of the curve), and synthesis rate (pool size $\times$ fractional turnover rate) were calculated for individual mice. Data are means ± standard deviation of $n = 5$ mice per group.
bile formation and gene expression patterns, kinetic studies were conducted in wild-type and $Fxr^{(-/-)}$ mice only. Analysis of plasma cholate enrichments over time (Figure 4) demonstrated that the cholate pool size, calculated from the y-intercept of the linear regression line shown in Figure 4, was larger in $Fxr^{(-/-)}$ mice than in wild-type mice (Figure 5A: $42 \pm 8 \mu$mol/100g vs. $23 \pm 3 \mu$mol/100g, $Fxr^{(-/-)}$ mice vs. wild-type; p < 0.0001). The percentage of cholate in hepatic bile, as determined by gas chromatographic analysis in individual mice (Table 3), was used to calculate total bile salt pool sizes. Under the assumption that all bile salt species displayed a similar cycling frequency, the calculated total pool sizes of non-cholate bile salts were similar ($22 \pm 4 \text{ mmol/100g vs. } 23 \pm 9 \text{ mmol/100g}, Fxr^{(-/-)}$ vs. wild-type mice, respectively, NS), leading to calculated total bile salt pool sizes of $64 \pm 12$ and $46 \pm 13 \text{ mmol/100g}$ in $Fxr^{(-/-)}$ and wild-type mice, respectively (p < 0.05). Deuterated cholate disappeared from plasma at the same rate in $Fxr^{(-/-)}$ and wild-type mice (Figure 4). The fractional turnover rate of cholate, calculated from the slope of the linear regression curve, was similar in

![Figure 5.](image)

**Figure 5.** Effects of FXR deficiency on pool size (A), fractional turnover rate (B), synthesis rate (C), cycling time (D), and daily intestinal reabsorption (E) of cholate as derived from $[^2H_4]$-cholate isotope enrichment measurements in plasma of wild-type and $Fxr^{(-/-)}$ mice. The pool size, fractional turnover rate (FTR), synthesis rate, cycling time and daily intestinal reabsorption were calculated in wild-type (white bars) and $Fxr^{(-/-)}$ (black bars) mice as described under Experimental Procedures. Data are means ± standard deviation of n = 5 mice per group. *Significant difference between wild-type and $Fxr^{(-/-)}$ mice.
both groups of mice. (Figure 5B: 0.5 ± 0.1 per day vs. 0.5 ± 0.2 per day, $Fxr^{(-/-)}$ vs. wild-type, respectively, NS).

In the $Fxr^{(-/-)}$ mice, the calculated cholate synthesis rate (Figure 5C) was two times increased compared to the wild-type mice (22 ± 2 mmol/100g/day vs. 11 ± 3 mmol/100g/day, $Fxr^{(-/-)}$ vs. wild-type mice; $p < 0.001$). In accordance with the increased cholate synthesis rate determined by stable isotope dilution, fecal loss of bile salts was increased by ~ 70 % (4.1 ± 1.1 mmol/day vs. 2.3 ± 0.7 mmol/day, $Fxr^{(-/-)}$ vs. wild-type mice; $p < 0.05$). The calculated cholate cycling time (Figure 5D) was not affected by FXR deficiency (4.4 ± 1.3 h vs. 4.3 ± 0.7 h, $Fxr^{(-/-)}$ mice vs. wild-type mice; NS). The calculated absolute amount of cholate reabsorbed in the intestines of $Fxr^{(-/-)}$ mice (Figure 5E) was ~ 2 fold larger than that in the intestines of wild-type mice (227 ± 81 mmol/100g/day vs. 121 ± 11 mmol/100g/day, $Fxr^{(-/-)}$ mice vs. wild-type mice; $p < 0.05$).

![Graph A](image)

**Figure 6.** mRNA and protein levels of intestinal bile salt transporters in wild-type and $Fxr^{(-/-)}$ mice. (A) Steady-state mRNA levels of *Asbt*, *Ibabp*, t-*Asbt*, and *Fic-1* in the ilea of wild-type (white bars) and $Fxr^{(-/-)}$ (black bars) mice. Total mRNA was isolated from the ileum, transcribed into cDNA, and subjected to real-time PCR as described under Experimental Procedures. *Significant difference between wild-type and $Fxr^{(-/-)}$ mice. (B) Western blot analysis of *Asbt* and *Ibabp* on brush-border membranes and liver homogenates, respectively, from the ilea of wild-type and $Fxr^{(-/-)}$ mice. Apparent molecular masses are indicated to the right.
Enterohepatic circulation of bile salts in farnesoid X receptor-deficient mice

Intestinal expression of genes involved in bile salt transport

To provide an explanation for the high bile salt absorption rate in Fxr(-/-) mice, the mRNA levels of several genes considered to be involved in intestinal bile salt absorption were determined in the terminal ileum. These studies showed that expression of Ibabp, a well known FXR target gene thought to be involved in intracellular bile salt trafficking and active bile salt reabsorption, was very strongly decreased at the mRNA level in Fxr(-/-) mice (Figure 6A). FXR deficiency did not affect expression of transporter protein-encoding genes Asbt, responsible for the major part of active ileal bile salt reabsorption, and of t-Asbt, putatively involved in basolateral bile salt efflux. Expression of Fic1 (Atp8b1), a P-type ATPase proposed to function as an aminophospholipid translocator and essential for normal bile salt metabolism, also did not differ at the mRNA level between wild-type and Fxr(-/-) mice. Western blot experiments on brush-border membrane fractions and homogenates of the terminal part of the ileum (Figure 6B) showed that Asbt protein levels were similar in wild-type and Fxr(-/-) mice, whereas the protein levels of Ibabp were essentially non-detectable in the Fxr(-/-) mice.

DISCUSSION

This study has established the physiological consequences of FXR deficiency on bile formation and on the kinetics of enterohepatic bile salt circulation employing an FXR-null mouse model generated by homologous recombination. A microscale stable isotope dilution technique was used to quantify important parameters of bile salt metabolism. Data show that the bile formation process per se was not affected by FXR deficiency and that effects on bile flow seen in Fxr(-/-) mice were secondary to alterations in bile salt metabolism. In accordance with current concepts of the role of FXR in control of bile salt synthesis, hepatic Cyp7a1 mRNA levels were significantly increased in Fxr(-/-) mice and were associated with an increased cholate synthesis rate. Enhanced bile salt synthesis was confirmed by increased fecal bile salt loss in Fxr(-/-) mice, although the absolute difference was somewhat less pronounced among the strains using this methodology. We attribute the discrepancy between outcome of fecal excretion and the isotope dilution method to the fact that no stool marker has been applied, which is required to correct for fecal balance measurements. Furthermore, the intrinsic difficulties of quantitative fecal bile salt analysis have been extensively reviewed by Setchell et al. As a consequence of defective feedback inhibition of hepatic bile salt synthesis, Fxr(-/-) mice developed an increased bile salt pool size, which implies that potential adaptive responses of intestinal bile salt reabsorption were not effective in maintenance of the bile salt pool size. No change in intestinal Asbt mRNA and protein levels was found in FXR-deficient mice. By contrast, the well known FXR target gene Ibabp was not expressed at all in the terminal ileum of Fxr(-/-) mice. Despite the absence of Ibabp, our kinetic study revealed that the absolute amount of bile salts reabsorbed from the intestine was not reduced, but was actually enhanced by 2-fold in Fxr(-/-) mice. These findings suggest that Ibabp may not function as a ‘facilitator’, but rather as a negative regulator of intestinal bile salt absorption under physiological conditions in the mouse.

FXR has been shown to be involved in control of various steps of bile
Chapter 5

salt metabolism, *i.e.*, synthesis and transport\(^{13,36,37,39}\), as well as in regulation of plasma lipoprotein metabolism\(^{2,3,13,40}\). FXR-deficient mice have been very informative in elucidation of the various functions of this nuclear bile salt-activated receptor. Studies by Sinal *et al.*\(^{13}\) and Lambert *et al.*\(^{40}\) were performed with FXR-deficient mice (C57BL/6J-SV129 background) that were generated by Cre-mediated deletion of a fragment containing the last exon of the *Fxr* gene, encoding the ligand-binding/dimerization domain, and the 3'-untranslated region of the *Fxr* mRNA. In theory, a truncated protein containing the DNA-binding domain could be formed that might affect expression of FXR target genes. In this study, we used an FXR knockout model (C57BL/6J-129/OlaHsd background) generated by homologous recombination, in which 292 bp of exon 2, encoding a part of the DNA-binding domain, were deleted. These mice showed plasma HDL and triglyceride levels (Elzinga *et al.*, unpublished) that were elevated to a similar extent as reported earlier\(^{13}\). In our study, liver function parameters were found to be unaffected in *Fxr*\(^{-/-}\) mice. Plasma bile salt concentrations were only slightly increased in *Fxr*\(^{-/-}\) mice compared with wild-type mice, in marked contrast to the 8-fold increase in plasma bile salt concentration in *Fxr*\(^{-/-}\) mice reported by Sinal *et al.*\(^{13}\). These strongly elevated plasma bile salt concentrations have been attributed to defective hepatobiliary bile salt transport due to down-regulation of the major canalicular bile salt export pump (Bsep)\(^{13}\). However, we have shown that a similar or even more pronounced down-regulation of Bsep expression in mice was not associated with impaired biliary bile salt secretion\(^{41}\). Furthermore, the more than 4-fold increase in biliary bile salt secretion during bile salt feeding in mice\(^{42}\) is accommodated by a very modest increase in hepatic Bsep expression. These data have been interpreted to indicate that Bsep at normal expression levels has a marked overcapacity in mice. In fact, in this study, biliary bile salt secretion was more than 2-fold increased despite a 40% reduction of *Bsep* expression. Therefore, it appears that the livers of *Fxr*\(^{-/-}\) mice are well able to handle the (increased) bile salt load. The discrepancy between both strains with respect to control of plasma bile salt concentrations remains unexplained at the moment.

FXR deficiency was associated with an enhanced bile flow, as determined during a 30 min period of bile collection. Biliary bile salt concentrations and secretion rates were clearly enhanced in *Fxr*\(^{-/-}\) mice, reinforcing the issue that decreased *Bsep* expression levels do not necessarily correlate with defective bile salt transport. The increase in bile flow in *Fxr*\(^{-/-}\) mice appeared to be exclusively due to the higher bile salt output, as is evident from the linear relationship between bile flow and biliary bile salt output that was obtained when data from wild-type, *Fxr*\(^{+/+}\) mice and *Fxr*\(^{-/-}\) mice were combined (Figure 2). The fact that values of individual mice from the three groups fitted well to this relationship indicates that the actual bile formation process was not affected by FXR deficiency. The value found for the choleretic activity (8 µl/µmol) of biliary bile salts is similar to that reported earlier in rodents\(^{43}\). The bile salt-independent fraction of bile flow (3 µl/min/100g body weight) was unaffected by FXR deficiency, which is in accordance with unchanged expression of *Mrp2*. Mrp2 is crucially involved in hepatobiliary transport of glutathione\(^{44}\), which represents the major driving force for the generation of bile salt-independent flow in rodents\(^{43,45}\). Although *Mrp2* has been identified as an FXR-target gene\(^{46}\), *Fxr*\(^{-/-}\) mice did not show reduced *Mrp2*
mRNA levels in this study or in a study by Schuetz et al.47. Biliary secretion rates of both cholesterol and phospholipids were slightly enhanced in Fxr(-/-) mice as compared to wild-type mice. Secretion of cholesterol and phospholipids into bile is coupled to that of bile salts33. Cholesterol secretion appears to involve the activity of Abcg5/Abcg8 dimers48,49, although the exact role of this twin transporter remains to be defined50, whereas phospholipid secretion critically depends on the activity of the Mdr2 P-glycoprotein51,52. Because expression of Abcg5/Abcg8 as well as of Mdr2 was unaffected in Fxr(-/-) mice (Figure 3B), it is plausible to ascribe the slight stimulation of biliary lipid secretion entirely to the enhanced bile salt secretion. It should be noted that Lambert et al.40 did report reduced hepatic Abcg5/Abcg8 expression in their strain of Fxr(-/-) mice, but this was found to be associated with increased biliary cholesterol output rates. The reason for the discrepancy in hepatic Abcg5/Abcg8 expression between both strains of mice is not clear.

We have focused on the effects of FXR deficiency on the kinetics of cholate metabolism. For this purpose, we used a novel microscale isotope dilution technique, applicable in unanesthetized animals21. FXR deficiency was associated with an increased cholate synthesis rate, in accordance with increased hepatic Cyp7a1 mRNA levels in Fxr(-/-) mice. Although FXR has been advocated as the major regulator of hepatic bile salt synthesis36,39, the effects of FXR deficiency on the basal expression of Cyp7a1 (~ +150%) and cholate synthesis (~ +67%) were relatively modest. This finding underscores the importance of the recently described FXR/SHP-independent mechanisms of regulation. Recent studies in bile salt-fed SHP knockout mice53,54 have clearly demonstrated the existence of FXR/SHP-independent repression of Cyp7a1 expression. Cyp27 and Cyp8b1 expression levels were not significantly affected in Fxr(-/-) mice, although the latter showed tendency to increase, in accordance with an increase in the fractional contribution of cholate in the bile salt pool of Fxr(-/-) mice. The fecal loss of bile salts was increased by ~70% in Fxr(-/-) mice. Because the mass of bile salts excreted into feces is, by definition, directly proportional to the amount synthesized in the liver36, the data on fecal loss confirm a generalized derepression of bile salt synthesis in FXR-deficient mice. This again is at variance with the study of Sinal et al.13, who reported increased expression of Cyp7a1, but decreased fecal bile salt loss in Fxr(-/-) mice.

The fractional turnover rate of cholate was similar in wild-type and Fxr(-/-) mice, whereas the cholate pool size was increased 2-fold in Fxr(-/-) mice, implying enhanced intestinal cholate reabsorption in Fxr(-/-) mice. The calculated total bile salt pool size was increased by ~40% in Fxr(-/-) mice. This is in contrast to the situation reported by Sinal et al.13, i.e., a reduction of the total bile salt pool size by ~50% in Fxr(-/-) mice fed a chow diet. In this case, bile salt pool size was measured in homogenates of gallbladder, the liver immediately surrounding the gall bladder, and the entire small intestine harvested after termination of the animals. The bile salt contents of the homogenates, which were extracted into ethanol, were determined colorimetrically. Whether methodological or strain-differences underlie the deviating results between both studies is not clear: the stable isotope dilution method is a well established procedure to quantify bile salt kinetics in humans55 and in laboratory animals21.
Maintenance of bile salt pool size can theoretically be regulated at the level of the intestine by controlled reabsorption in the terminal ileum. Asbt had been identified as the major transporter involved in this process. However, expression of Asbt was not different between wild-type and Fxr\(^{(-/-)}\) mice. In contrast, Chen et al.\(^{17}\) reported an increase in Asbt protein levels in Fxr\(^{(-/-)}\) mice, in accordance with the presence of LRH-1 sites in the promoter of the murine Asbt gene identified by these authors. t-Abst and Fic1 expression was also not changed at the mRNA level between wild-type and Fxr\(^{(-/-)}\) mice and therefore does not seem to play a role in enhanced bile salt reabsorption efficiency. Ibabp, a well known FXR gene, was drastically down-regulated at mRNA and protein level in the ilea of Fxr\(^{(-/-)}\) mice, in accordance with earlier studies\(^{13,17}\). Ibabp is thought to be involved in intracellular bile salt trafficking and to facilitate reuptake of bile salts in the small intestine\(^{8,9}\). Yet despite the complete absence of Ibabp protein in the ilea of the Fxr\(^{(-/-)}\) mice, daily intestinal cholate reabsorption was much higher than in wild-type mice. This suggests that, under physiological conditions, Ibabp functions as a negative rather than as a positive regulator of intestinal bile salt reabsorption in the mouse.

In conclusion, this work shows that the absence of FXR in vivo in mice is associated with defective feedback inhibition of hepatic cholate synthesis, which leads to an enlarged circulating cholate pool with an unaltered fractional turnover rate. The absence of Ibabp does not negatively interfere with the enterohepatic circulation of cholate in mice.

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