Chapter 7

Effects of pharmacological FXR activation on the enterohepatic circulation of bile salts in rats

Inhibition of cholate synthesis rate and reduced cholate pool size despite increased expression of the ileal bile acid-binding protein

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Submitted
The bile salt-activated farnesoid X receptor (FXR; NR1H4) controls transcription of various genes involved in bile salt synthesis and transport. We studied the physiological consequences of prolonged treatment of rats with the FXR agonist GW4046 (25 mg. kg\(^{-1}\).day\(^{-1}\) for 6 days) on the kinetics of the enterohepatic cycling of cholate, the major bile salt species in rats. Pool size, fractional turnover rate, synthesis rate and cycling time of cholate were determined by stable isotope dilution and related to expression of relevant hepatic and intestinal transporters in control and GW4046-treated rats. Cholate synthesis rate was decreased by 50%, which was associated with a reduction of the cholate pool size by 45% upon GW4046 treatment. Reduced cholate synthesis coincided with a ~50% decrease in hepatic sterol 12\(\alpha\)-hydroxylase (Cyp8b1) expression, whereas cholesterol 7\(\alpha\)-hydroxylase (Cyp7a1) expression was not significantly affected. Protein and mRNA expression of the ileal bile acid-binding protein (Ibabp), but not of the apical sodium-dependent bile salt transporter (Asbt), were increased with a shift towards more proximal parts of the ileum upon FXR activation. Despite this, fractional turnover rate and cycling time of the cholate pool as well as the calculated amount of cholate lost from the intestine per day were not significantly affected. Thus, treatment with the FXR agonist GW4046 in rats is associated with inhibition of the cholate synthesis rate and reduction of the cholate pool size. Induction of intestinal Ibabp expression does not contribute to the maintenance of the pool size in this condition of impaired bile salt synthesis.
Bile salts serve a number of important functions in the body. Hepatobiliary excretion of bile salts provides the main driving force for the generation of bile flow\(^1\). Secondly, due to their detergent properties, bile salts aid in the solubilization of biliary lipids (phospholipids, cholesterol) and facilitate intestinal absorption of dietary fats including fat-soluble vitamins\(^1\). Thirdly, bile salts participate in regulation of pancreatic enzyme activities and the release of cholecystokinin\(^2,3\). Finally, bile salts are essential in cholesterol homeostasis: conversion of cholesterol into bile salts and their subsequent fecal excretion provides the major route for elimination of excess cholesterol\(^1\).

Recently, it has become clear that bile salts also act as signaling molecules. Bile salts exert regulatory actions on expression of specific genes via activation of a nuclear receptor, i.e., the farnesoid X receptor (FXR; NR1H4)\(^5\). Bile salt-activated FXR controls expression of several genes considered crucial in maintenance of bile salt and cholesterol homeostasis\(^4,6\). Activated FXR inhibits transcription of the \textit{Cyp7a1} gene, encoding cholesterol 7alpha-hydroxylase which catalyzes the first and rate-limiting step in bile salt synthesis\(^7\). This repression is achieved indirectly via a coordinated regulatory cascade involving other liver-specific factors, including small heterodimer partner (SHP; NROB2). FXR-induced expression of SHP inhibits the activity of the tissue-specific factor liver receptor homologue-1 (LRH-1; NR5A2), which controls expression of \textit{Cyp7a1}\(^8\). Sterol 12alpha-hydroxylase (Cyp8b1), the enzyme that controls the ratio in which the primary bile salt species cholate and chenodeoxycholate are being formed, is also negatively controlled by bile salts in an FXR-dependent manner\(^9\). Furthermore, activated FXR controls expression of hepatic bile salt transporters, i.e., it directly induces the expression of Bsep (Abcb11)\(^10,11\) and down-regulates the expression of Na+-taurocholate co-transporting polypeptide (Ntcp; Slc10a1) via SHP\(^12\). Two intestinal proteins are considered to facilitate intestinal bile salt reabsorption; the apical sodium-dependent bile salt transporter (Asbt; Slc10a2) and the ileal bile acid-binding protein (Ibabp)\(^13\). Chen \textit{et al.}\(^14\) demonstrated that murine Asbt appears to be subjected to negative feedback regulation mediated by FXR activation in a species-dependent manner, although recent studies from our lab in FXR-deficient mice did not confirm this\(^15\). Intestinal expression of Ibabp, a cytosolic protein related to L-FABP and considered to be involved in intracellular trafficking of bile salts, is strongly induced by bile salts via FXR\(^5,16,17\). In agreement with this concept, we and others have shown that FXR-deficiency in mice results in a strong downregulation of Ibabp mRNA and protein levels\(^15\). It has also been demonstrated that FXR regulates a variety of genes involved in control of plasma lipid levels\(^16-20\). Consequently, FXR may represent a novel target for pharmacological manipulations of plasma lipoprotein levels aimed at treatment or prevention of cardiovascular disease.

In view of the broad spectrum of genes controlled by FXR, it is essential to understand the impact of pharmacologically induced alterations in FXR activity, not only on expression of individual genes, but also on physiological parameters. We have evaluated the effects of prolonged treatment with the FXR agonist GW4046 on whole body kinetics of the enterohepatic circulation of cholate, the major primary bile salt in rats. Pool size, fractional turnover rate, synthesis rate and intestinal absorption of cholate were determined by stable isotope dilution and related to expression of relevant transporters in liver and intestine. Treatment with the FXR agonist GW4046 in rats was found to be associated with a marked inhibition of the cholate synthesis rate. This resulted in a diminished circulating cholate
pool with an unaltered fractional turnover rate. FXR induced expression of intestinal Ibabp apparently did not enhance bile salt reabsorption to maintain pool size under these conditions of impaired bile salt synthesis.

EXPERIMENTAL PROCEDURES

Animals
Male Wistar rats (Harlan Laboratories, Zeist, The Netherlands, mean body weight ± SD: 324 ± 24 g), were kept in a light-and temperature-controlled environment. They were fed standard rodent diet (RMH-B, Hope Farms BV, Woerden, The Netherlands) and tapwater ad libitum. Experimental protocols were approved by the local Ethical Committee for Animal Experiments.

Materials
[2,2,4,4-2H]-cholate (2H4-cholate, isotopic purity 98%) was obtained from Isotech Inc. (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
Rats were equipped with a permanent heartcatheter under halothane anesthesia as described previously21. After a recovery period of 2 days, rats were treated with the FXR agonist GW4046 (dose 25 mg.kg-1.day-1) or with its solvent (DMSO 0.5%; cremophor 0.5%; 5% water/mannitol) by gavage for 6 days. GW4046 was kindly provided by Dr. B. Shan and Dr. M. Schwarz (Tularik Incorporated, South San Francisco, CA). At day 6, 2H4-cholate (5 mg/rat) was intravenously administered to GW4046-treated and control rats. Blood samples (0.25 ml) were collected before and at 12, 24, 36 and 48 hours after administration of 2H4-cholate. Plasma was obtained by centrifugation at 9000 rpm for 10 minutes and stored at -20 °C until analyzed. At day 8, animals were anaesthetized by intraperitoneal injection of Hypnorm (1 ml/kg) and Diazepam (10 mg/kg) (Janssen Pharmaceutica, Beerse, Belgium) and, after collection of a single 15-minute bile sample via a bile fistula, the liver and small intestine were removed. The 30 cm distal end of the small intestine was rinsed with cold phosphate-buffered saline containing phenyl-methyl-sulfonylfluoride (PMSF) to prevent protein degradation and divided in proximal, medial and distal segments of 10 cm. Tissue samples were immediately frozen in liquid nitrogen and stored at - 80 °C for membrane preparation and RNA isolation.

Analytical procedures
Plasma alanine transaminase (ALAT), aspartate transaminase (ASAT) and bilirubin were determined by routine laboratory techniques. Total bile salts in plasma and bile were determined by an enzymatic fluorimetric assay using 3α-hydroxysteroid dehydrogenase22. Plasma triglycerides, HDL cholesterol and total cholesterol were determined using commercially available kits (Roche Molecular Biochemicals, Mannhein, Germany). Levels of biliary cholesterol and phospholipids were measured as described by Kuipers et al.21.
Gas chromatography
Bile salt composition of bile samples was determined by capillary gas chromatography as methyl ester/trimethylsilyl derivatives on a Hewlett Packard gas chromatograph (HP 5880 A), equipped with a 50 m x 0.32 mm, CP-Sil-19 fused silica column (Chrompack BV, Middelburg, The Netherlands).

Gas-liquid chromatography/electron capture negative chemical ionization mass spectrometry
Plasma samples were prepared for bile salt analysis by gas chromatography mass spectrometry (GC-MS) as described by Hulzebos et al. All analyses were performed on a Finnigan SSQ7000 Quadrupole GC-MS instrument (Finnigan MAT, San José). GC separation was performed on a 15 m x 0.25 mm column, 0.25 µm film thickness (AT-5MS, Alltech Associates Inc., Deerfield, IL).

Western blotting
Intestinal brush border membranes were isolated as described by Schmitz et al. Total protein concentration of membrane fractions was determined using the method described by Lowry et al. Separation of proteins was performed on 4-15% gradient gels (BioRad, Hercules, USA) and proteins were transferred to ECL-Hybond nitrocellulose (Amersham Biosciences, Buckinghamshire, United Kingdom) by Western blotting. Ibabp protein content of intestinal homogenates and Asbt protein content of brush border membranes, were determined using recombinant anti-murine Ibabp antibody and polyclonal anti-hamster Asbt antibody, respectively. Detection of immune complexes in intestinal membranes was performed using anti-rabbit antibody linked to horse radish peroxidase (Sigma, St. Louis, MO) as secondary antibody and enhanced chemiluminescence as provided by the manufacturers (ECL, Amersham Biosciences).

RNA isolation and PCR procedures
Total RNA from liver and the three intestinal sections per animal was isolated and quantified using Ribogreen (Molecular Probes, Inc., Leiden, The Netherlands). cDNA synthesis was done as previously described. Primers were obtained from Invitrogen (Carlsbad, USA). Fluorogenic probes, labeled with 6-carboxy-fluorescein (FAM) and 6-carboxy-tetramethyl-rhodamine (TAMRA), were made by Eurogentec (Seraing, Belgium). Primers and probes 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 Bsep (Abcb11), Cyp7a1, Cyp27, and Ntcp (Slc10a1) have been described by Plösch et al. Primer and probes for Mrp2 (Abcc2) Cyp8b1, Shp (Nr0b2), Fic1 (Atp8b1) and Mrp3 (Abcc3) have been described by Kok et al. Primers and detection probes for the gene of interest, labeled with a fluorescent reporter dye (6-carboxy-fluorescein) and a fluorescent quenching dye (6-carboxy-tetramethyl-rhodamine), were added. Fluorescence was measured by an ABI Prism 7700 Sequence Detector v. 1.6 software (Perkin-Elmer Corp., Foster City, CA). All expression data were subsequently standardized for β-actin, which was analyzed in separate runs.

Isotope dilution technique calculations
The area ratio M4-CA/ M0-CA was calculated as described by Hulzebos et al., using LCQuan software (Finnigan Corp., San José). Enrichment was defined as the increase of...
M₄-CA/ M₀-CA relative to baseline measurements after administration of ²H₄-CA and expressed as the natural logarithm of atom % excess (ln APE) value. The decay of ln APE in time was calculated by linear regression analysis. From this linear decay curve the fractional turnover rate (FTR) and pool size of CA were calculated. The FTR (day⁻¹) equals the slope of the regression line. The pool size (µmol.100g⁻¹) 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. Cholate synthesis rate (µmol.100g⁻¹.day⁻¹) 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 (µmol. 100g⁻¹) by the biliary secretion rate of cholate (µmol.100g⁻¹.h⁻¹). The biliary secretion of cholate was calculated by multiplying the bile flow (µl.100g⁻¹.h⁻¹) with the cholate concentration (mM) in a single 15 minute bile fraction, obtained immediately after canulation of the common bile duct. The amount of cholate reabsorbed per day was calculated by multiplying pool size and cycling frequency (cycling frequency is equal to 24/cycling time (h)) and subtracting daily synthesis rate. The fraction of cholate lost per enterohepatic cycle was calculated by dividing cholate synthesis rate by cholate cycling frequency and was expressed as percentage of total cholate pool size, assuming steady state conditions in which synthesis rate equals fecal loss.

**Statistical analysis**

All results are presented as means ± standard deviation. Differences between GW4046-treated and control rats were evaluated by Mann Whitney U-test. Level of significance for statistical analyses was set at \(p<0.05\). Analysis was performed using SPSS for Windows software (SPSS Inc., Chicago, IL).

**RESULTS**

**Animal characteristics and effects of treatment with the FXR agonist GW4046 on liver function parameters and plasma lipid levels**

Treatment with the FXR agonist GW4046 did not affect body weights of the rats (Table 1). The liver weight/body weight ratio was also unaffected upon treatment. There were no significant differences in aspartate and alanine transaminase activities nor in plasma bilirubin concentrations between GW4046-treated and control rats (Table 1). On the treatment protocol employed, plasma bile salt concentrations were somewhat decreased in the GW4046-treated rats. Levels of plasma HDL cholesterol were slightly, but significantly elevated, whereas total cholesterol levels remained unaffected. Plasma triglycerides were somewhat lower after treatment with GW4046, but the difference between treated and control rats did not reach statistical significance.

**Effects of GW4046 treatment on kinetic parameters of cholate metabolism**

Figure 1 shows plasma cholate enrichments over time and demonstrates that deuterated cholate disappeared from plasma at the same rate after treatment with the FXR agonist GW4046 or with its solvent. The cholate pool size (Figure 2A), calculated from the y-intercept of the linear regression line (Figure 1), was reduced by ~45% after treatment.
Enterohepatic circulation of bile salts in FXR ligand-treated rats

With the FXR agonist (13 ± 2 µmol.100g⁻¹ vs. 24 ± 5 µmol.100g⁻¹, GW4046-treated rats vs. controls; p<0.01). The fractional turnover rate of cholate (Figure 2B), calculated from the slope of the linear regression curve (Figure 1), was similar in GW4046-treated rats and controls (0.34 ± 0.06 pools.day⁻¹ vs. 0.37 ± 0.07 pools.day⁻¹, respectively). GW4046 decreased the cholate synthesis rate (Figure 2C) by ~ 50 % compared with controls (4.5 ± 0.6 µmol.100g⁻¹.day⁻¹ vs. 8.5 ± 0.6 µmol.100g⁻¹.day⁻¹, GW4046-treated rats vs. controls; p<0.001). The cholate cycling time (Figure 2D) was not affected upon treatment with the

Table 1. Animal characteristics, parameters of liver function and plasma lipids

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GW4046</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>320 ± 29</td>
<td>329 ± 18</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12 ± 1.0</td>
<td>12 ± 0.5</td>
</tr>
<tr>
<td>Liver/body weight ratio</td>
<td>0.037 ± 0.002</td>
<td>0.036 ± 0.001</td>
</tr>
<tr>
<td>Bile salts (µmol/L)</td>
<td>50 ± 31</td>
<td>29 ± 30</td>
</tr>
<tr>
<td>Total bilirubin (µmol/L)</td>
<td>7.3 ± 1.2</td>
<td>7.2 ± 0.8</td>
</tr>
<tr>
<td>Alanine transaminase (U/L)</td>
<td>85 ± 113</td>
<td>43 ± 6</td>
</tr>
<tr>
<td>Aspartate transaminase (U/L)</td>
<td>117 ± 105</td>
<td>71 ± 17</td>
</tr>
<tr>
<td>Cholesterol (mmol/L)</td>
<td>1.8 ± 0.2</td>
<td>1.8 ± 0.2</td>
</tr>
<tr>
<td>HDL cholesterol (mmol/L)</td>
<td>1.1 ± 0.1</td>
<td>1.3 ± 0.1*</td>
</tr>
<tr>
<td>Triglycerides (mmol/L)</td>
<td>0.76 ± 0.27</td>
<td>0.53 ± 0.14</td>
</tr>
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</table>

Male Wistar rats were treated with GW4046 or solvent only for 6 days. GW4046 was administered orally at a dose of 25 mg.kg⁻¹.day⁻¹. Means ± SD of control and GW4046-treated rats (n = 5-6 per group). The asterisk indicates significant difference (Mann-Whitney U test, p < 0.05).

Figure 1. Decay of intravenously administered [²H₄]-cholate in male Wistar rats treated with the FXR agonist GW4046 or its solvent. A dose of 5 mg of [²H₄]-cholate was intravenously injected in male Wistar rats treated with GW4046 (closed bars) or solvent only (open bars). Blood samples were collected at 12, 24, 36, and 48h after injection for determination of plasma cholate enrichments by GC-MS as described under Experimental procedures. Data represent mean ± standard deviations of n = 4 rats per group. The asterisks indicate significant difference (Mann-Whitney U test, p < 0.05).
FXR agonist (2.0 ± 1.1 h vs. 2.1 ± 0.6 h, GW4046-treated rats vs. controls; NS). Consequently, the calculated absolute amount of cholate lost per cycle was decreased upon GW4046 treatment (0.34 ± 0.15 µmol.100g⁻¹ vs. 0.73 ± 0.21 µmol.100g⁻¹, GW4046-treated rats vs. controls; p<0.05). The calculated amount of cholate reabsorbed per day tended to be reduced upon treatment, but the difference between both groups did not reach statistical significance (Figure 2E) (196 ± 94 µmol.100g⁻¹.day⁻¹ vs. 293 ± 94 µmol.100g⁻¹.day⁻¹, GW4046-treated rats vs. controls; NS).

Effects of GW4046 treatment on steady-state hepatic mRNA levels of genes involved in bile salt synthesis and bile salt transport
Figure 3 shows hepatic expression of specific genes, as influenced by treatment with the FXR agonist GW4046, determined by real-time quantitative PCR. Expression levels of Fxr and Shp were unaffected upon treatment with GW4046. Levels of Cyp7a1 mRNA tended to decrease upon treatment, but, like those of Cyp27, were not significantly affected. In contrast, mRNA levels of Cyp8b1 were significantly decreased. Moreover, mRNA levels of the gene encoding the canalicular bile salt transporter Bsep, a well-known FXR target gene, were significantly increased upon treatment with GW4046. Expression of other transporters relevant to bile salt uptake and bile formation, like Ntcp and Mrp2, were not changed by treatment with the FXR agonist.
Enterohepatic circulation of bile salts in FXR ligand-treated rats

Table 2. Biliary bile salt* composition in male Wistar rats after treatment with the FXR agonist GW4046 or its solvent

<table>
<thead>
<tr>
<th>Strain</th>
<th>α-MC</th>
<th>β-MC</th>
<th>Δ22β-MC</th>
<th>C</th>
<th>CDC</th>
<th>HDC</th>
<th>DC</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>4 ± 1</td>
<td>4 ± 2</td>
<td>10 ± 3</td>
<td>66 ± 2</td>
<td>4 ± 1</td>
<td>6 ± 2</td>
<td>4 ± 2</td>
</tr>
<tr>
<td>GW4046</td>
<td>4 ± 1</td>
<td>6 ± 3</td>
<td>11 ± 5</td>
<td>56 ± 5*</td>
<td>7 ± 1*</td>
<td>9 ± 2</td>
<td>4 ± 1</td>
</tr>
</tbody>
</table>

Male Wistar rats were treated with GW4046 or solvent only for 6 days. GW4046 was administered orally at a dose of 25 mg.kg⁻¹.day⁻¹. α-MC = α-Muricholate, β-MC = β-Muricholate, Δ22β-MC = Δ22β-Muricholate, C = Cholate, CDC = Chenodeoxycholate, HDC = Hyodeoxycholate, DC = Deoxycholate. * >90% of all bile salt species represented. Values are expressed as a percentage of the total amount. Means ± SD of control and GW4046-treated rats (n = 5-6 per group). The asterisks indicate significant difference (Mann-Whitney U test, p < 0.05).

Effects of GW4046 treatment on bile formation and bile composition

Total biliary bile salt concentration was similar in both groups (53 ± 18 mmol/l vs. 41 ± 16 mmol/l, GW4046-treated rats vs. controls; NS). As expected from reduced Cyp8b1 expression, the cholate fraction in bile was decreased and the relative proportion of chenodeoxycholate was significantly increased upon treatment with GW4046 (Table 2). GW4046 treatment did not affect bile flow rate after interruption of the enterohepatic circulation (Table 3). Despite the observed increase in hepatic mRNA levels of Bsep, total biliary bile salt secretion rate measured during the first 15 minutes after initiation of bile collection was not significantly altered upon GW4046 treatment. Biliary phospholipid and cholesterol output rates, which are coupled to secretion of bile salts, were also similar in both groups.
### Table 3. Bile flow and biliary output rates in male Wistar rats treated with the FXR agonist GW4046 or its solvent

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>GW4046</th>
</tr>
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<tbody>
<tr>
<td><strong>Bile flow (µl/min/100 g BW)</strong></td>
<td>5.9 ± 0.3</td>
<td>6.5 ± 0.6</td>
</tr>
<tr>
<td><strong>Biliary output (nmol/min/100 g BW)</strong></td>
<td></td>
<td></td>
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<tr>
<td>Bile salt output</td>
<td>313 ± 92</td>
<td>268 ± 109</td>
</tr>
<tr>
<td>Cholate output</td>
<td>209 ± 66</td>
<td>151 ± 63</td>
</tr>
<tr>
<td>Phospholipid output</td>
<td>30.6 ± 2.9</td>
<td>32.2 ± 11.3</td>
</tr>
<tr>
<td>Cholesterol output</td>
<td>3.37 ± 1.10</td>
<td>3.35 ± 0.95</td>
</tr>
</tbody>
</table>

Male Wistar rats were treated with GW4046 or solvent only for 6 days. GW4046 was administered orally at a dose of 25 mg.kg⁻¹.day⁻¹. Means ± SD of control and GW4046-treated rats (n = 3-5 per group). BW = body weight.

### Figure 4. Intestinal mRNA expression levels of genes involved in intestinal bile salt transport of male Wistar rats treated with the FXR agonist GW4046 or its solvent measured by real-time PCR.

Steady-state mRNA levels of Asbt, Fic1, tAbst, Mrp3, Fxr and Shp. Male Wistar rats were treated with GW4046 (closed bars) or solvent only (open bars) for 6 days (n = 4 per group); the 30 cm distal end of the small intestine was removed, rinsed with cold phosphate-buffered saline, divided into three equal parts, and analyzed as described under Experimental procedures. All data were standardized for β-actin. Expression of mRNA in the proximal part of the small intestine in rats receiving the solvent only was set to 1.

### Effects of GW4046 treatment on steady-state intestinal mRNA levels and protein expression of Ibabp

The mRNA levels of genes encoding proteins involved in intestinal bile salt transport were measured in different sections (proximal, medial and distal) of the terminal part (30 cm) of the small intestine. Figure 4 shows that most genes were lowly expressed in the proximal segment and showed a steep increase towards the distal ileum. Treatment of rats with GW4046 did not affect mRNA levels of Asbt, Fic1 (both putatively involved in bile salt uptake³⁰,³¹), tAbst and Mrp3 (both putatively involved in basolateral efflux³⁰). A similar
expression pattern was observed for \( Fxr \) and \( Shp \). GW4046 treatment caused a huge increase in \( Ibabp \) expression in the proximal and medial parts of the ileum (Figure 5A), i.e., segments in which \( Ibabp \) expression was low in control rats. In agreement with this, an increase in \( Ibabp \) protein levels was observed upon treatment with GW4046 (Figure 5B). In control rats, \( Ibabp \) protein level was exclusively detectable in the distal part, while after GW4046 treatment \( Ibabp \) protein was also clearly present in the medial segments. \( Asbt \) protein levels varied among individual animals of the same group, but was not significantly changed between treated and control rats (data not shown).

**Figure 5.** \( Ibabp \) mRNA and protein expression in the small intestine treated with the FXR agonist GW4046 or its solvent measured by real-time PCR and Western blot. Wistar rats were treated with GW4046 (closed bars) or solvent only (open bars) for 6 days (\( n = 4 \) per group); the 30 cm distal end of the small intestine was removed, rinsed with cold phosphate-buffered saline, and analyzed as described under Experimental procedures. (A) Steady-state \( Ibabp \) mRNA levels (insert) and relative GW4046-induced changes in \( Ibabp \) expression in subsequent ileal sections (in which each section in rats receiving the solvent was set to 1) and (B) \( Ibabp \) protein levels in ileum. Results are shown for three sequential distal brush border membrane fractions per animal, and for two animals per group that are representative for \( n = 4 \) per group. The asterisks indicate significant difference (Mann-Whitney \( U \) test, \( p < 0.05 \)). \( P \) = proximal, \( M \) = medial and \( D \) = distal.

**DISCUSSION**

To evaluate the physiological consequences of pharmacological FXR activation by GW4046 on the enterohepatic circulation of bile salts, kinetic parameters of cholate metabolism were determined using a recently developed stable isotope dilution method \(^{23} \). In accordance with current concepts of the role of FXR in control of bile salt synthesis\(^{4,32} \), cholate synthesis rate was \( \sim 2 \)-fold reduced upon FXR activation by treatment with GW4046 in rats. Impaired cholate synthesis coincided with a 50% decrease in mRNA levels of hepatic \( Cyp8b1 \), encoding sterol 12\( \alpha \)-hydroxylase, which regulates the ratio in which the primary bile salts cholate and chenodeoxycholate are being formed\(^4 \). Accordingly, the amount of cholate in bile was reduced relative to that of chenodeoxycholate upon treatment with the FXR agonist. \( Cyp7a1 \) mRNA expression levels tended to be decreased after treatment with GW4046. The absence of significantly reduced \( Cyp7a1 \) mRNA levels may be related to several factors. Expression of \( Cyp7a1 \) mRNA is subjected to a strong circadian rhythm and exhibits greatest expression in the dark phase. Harvesting of liver was performed \( \sim 4 \) h into the light phase, i.e., when expression is low, and this could possibly contribute to the absence of
significant effects on \textit{Cyp7a1} expression\textsuperscript{33,34}. Under the conditions employed, expression of \textit{Shp}, putatively involved in transcriptional regulation of \textit{Cyp7a1}, was also unaffected. From recent studies is known that \textit{Cyp7a1} expression is regulated via SHP-dependent as well as via SHP-independent pathways\textsuperscript{35-37}. Our data delineate the fact that hepatic \textit{Cyp7a1} mRNA expression levels not always reflect bile salt synthesis rate in rats.

Upon treatment with GW4046, the cholate pool size was reduced, probably related to FXR induced reduction of the cholate synthesis rate. Under steady state conditions, only a relatively small fraction of bile salts escapes intestinal absorption and is lost into the feces, which is compensated for by \textit{de novo} bile salt biosynthesis in the liver. Apart from its central role in control of hepatic bile salt synthesis, FXR is supposedly also involved in adaptive regulation of the expression of intestinal bile salt transporters in response to changes of the bile salt pool. Various studies indicate that regulation of the bile salt pool size may not only occur at the level of hepatic biosynthesis in response to intestinal events, but that intestinal events can influence the bile salt pool size independently\textsuperscript{38}. We have demonstrated previously that cyclosporin A treatment markedly reduces cholate synthesis rate in rats, but does not affect cholate pool size. The maintenance of cholate pool size was associated with increased Asbt protein expression in the distal ileum\textsuperscript{29}. In analogy with this finding, disruption of the \textit{Slc10a2} gene, encoding Asbt, results in an increased bile salt synthesis in the presence of a decreased bile salt pool size in \textit{Asbt} \textsuperscript{-/-} mice\textsuperscript{39}. In the current study, administration of the FXR agonist had no effect on mRNA expression of \textit{Asbt} localized in the terminal ileum. Asbt protein expression varied among individual animals of the same group, but was not significantly changed upon treatment. The unchanged expression of \textit{Asbt} is in agreement with Chen \textit{et al.}\textsuperscript{14}, who reported that rat Asbt is not under control of FXR. In contrast, expression of the well-known FXR target gene \textit{Ibabp}, considered to be involved in the intracellular trafficking of bile salts, was increased in the terminal ileum and this protein expression shifted towards more proximal segments upon GW4046-treatment. Yet, these intestinal adaptations did not fully compensate for the impaired bile salt synthesis, since chronic FXR activation resulted in a smaller bile salt pool size. In fact, these results are in line with previous data obtained by our group in FXR-deficient mice, in which \textit{Ibabp} protein was undetectable, but the absolute amount of cholate reabsorbed by the intestine was increased rather than decreased\textsuperscript{15}. Together these data imply that the presumed role of \textit{Ibabp} in bile salt transport\textsuperscript{5,17} needs revision and that clinical application of selective FXR-ligands resulting in reduced bile salt synthesis and pool size may be beneficial in treatment of cholestatic liver disease.

Our data show that the bile formation process \textit{per se} was not affected upon treatment with GW4046 in rats. No changes occured in the biliary output of cholesterol, phospholipids or bile salts. Biliary bile salt output was unchanged despite the significant increase of mRNA levels of \textit{Bsep}, a well-known target gene of FXR. It has been postulated that \textit{Bsep}, at normal expression levels, has already a marked overcapacity, at least in mice. For example, a more than 4-fold increase in biliary bile salt secretion during bile salt feeding in mice was accommodated by a very modest increase in hepatic \textit{Bsep} expression\textsuperscript{40}. Thus, considering the constancy of bile salt secretion, the \textasciitilde 50\% increase of \textit{Bsep} expression is probably a direct consequence of treatment with the FXR agonist. Although \textit{Mrp2} has been identified as an FXR target gene\textsuperscript{41}, \textit{Mrp2} mRNA levels were unaffected upon treatment with GW4046. This is in agreement with previous studies, showing that FXR-deficiency in mice does not affect hepatic \textit{Mrp2} mRNA levels\textsuperscript{15,42,43}.

Apart from its central role in bile salt metabolism, FXR plays an important role in
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control of plasma lipid concentrations\textsuperscript{19,20,44}. We and others have shown that $FXR^{-/-}$ mice exhibit increased plasma lipid concentrations (Elzinga et al., unpublished data)\textsuperscript{9}. Development of selective ligands for FXR could therefore, in theory, contribute to novel therapeutic approaches to treat hyperlipidemia. Upon treatment with the FXR agonist GW4046, at a dose of 25 mg.kg\textsuperscript{-1}.day\textsuperscript{-1} for 6 days, triglyceride levels were reduced by 30%. Although statistically non-significant, a decrease of this magnitude can still be relevant in clinical practice. In agreement with our finding is the dose-dependent decrease of plasma triglyceride levels reported in Fischer rats treated with the same FXR ligand\textsuperscript{45}. Administration in a dose of 30 mg.kg\textsuperscript{-1}.day\textsuperscript{-1} and 100 mg.kg\textsuperscript{-1}.day\textsuperscript{-1} for 7 days decreased triglyceride levels by $\sim 25\%$ (non-significant) and by $\sim 50\%$, respectively. Although total plasma cholesterol levels were not affected by treatment with GW4046, plasma HDL cholesterol increased after FXR activation, in agreement with other studies\textsuperscript{45}. However, FXR-deficiency in mice on a regular chow diet also leads to raised plasma HDL cholesterol levels (Elzinga et al., unpublished data)\textsuperscript{46}. Furthermore, it has been demonstrated that activated FXR is a negative regulator of human apoA-I expression and FXR activation is associated with reduced plasma concentrations of apoA-I and serum HDL-cholesterol levels in mice\textsuperscript{19}. In our study, treatment of rats with the FXR agonist GW4046 did not affect hepatic mRNA expression of $ApoA-I$ (data not shown). The reason for elevated HDL levels upon GW4046 treatment in rats remains elusive at this moment. Species-differences in the FXR-mediated control of ApoA-I may contribute to these divergent results.

In conclusion, treatment with the FXR agonist GW4046 in rats is associated with increased plasma HDL cholesterol levels, inhibition of the cholate synthesis rate and reduction of the cholate pool size. Induction of intestinal $Ibap$ expression does not enhance bile salt reabsorption efficiency to maintain pool size under this condition of impaired bile salt synthesis.

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Chapter 7

REFERENCES

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