Chapter 3

Cyclosporin A and enterohepatic circulation of bile salts in rats: decreased cholate synthesis but increased intestinal reabsorption

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ABSTRACT

Background/Aims: Cyclosporin A (CsA) has been shown to inhibit synthesis and hepatobiliary transport of bile salts. However, effects of CsA on the enterohepatic circulation of bile salts in vivo are largely unknown. We characterized the effects of CsA on the enterohepatic circulation of cholate, with respect to synthesis rate, pool size, cycling time, intestinal absorption, and the expression of relevant transporters in liver and intestine in rats.

Methods: CsA (1 mg·100 g⁻¹·day⁻¹ s.c.) or its solvent was administered daily to male rats for 10 days. Cholate synthesis rate and pool size were determined by a [²H₄]-cholate dilution technique. Bile and feces were collected for determination of cholate and total bile salts, respectively. Cycling time and intestinal absorption of cholate were calculated. The mRNA levels and corresponding transporter protein levels in liver and intestine were assessed by real-time polymerase chain reaction and Western analysis, respectively.

Results: CsA treatment decreased cholate synthesis rate by 71%, but did not affect pool size or cycling time. CsA reduced the amount of cholate lost per enterohepatic cycle by ~70%. Protein levels of the apical sodium-dependent bile salt transporter (Asbt) were 2-fold increased in distal ileum of CsA-treated rats, due to post-transcriptional events.

Conclusions: Chronic CsA treatment markedly reduces cholate synthesis rate in rats, but does not affect cholate pool size or cycling time. Our results strongly suggest that CsA enhances efficacy of intestinal cholate reabsorption through increased Asbt protein expression in the distal ileum, which contributes to maintenance of cholate pool in CsA-treated rats.
INTRODUCTION

Bile formation is mainly driven by active hepatobiliary secretion of bile salts mediated by the canalicular bile salt export pump (Bsep or Abcb11 according to new nomenclature), a member of the P-glycoprotein subfamily of ATP-binding cassette transporters. After secretion into the bile, the majority of bile salts is maintained within the enterohepatic circulation, implying reabsorption from the intestine and reuptake by the liver. Intestinal absorption of bile salts is, to a large extent, mediated by the apical sodium-dependent bile salt transporter (Asbt) localized in the terminal ileum. After their intestinal uptake and subsequent appearance in the portal circulation, bile salts are efficiently taken up by hepatocytes, mainly via the Na+-taurocholate cotransporting polypeptide (Ntcp) and organic anion transporting polypeptide (Oatp). 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 de novo bile salt biosynthesis in the liver. Thus, the size of bile salt pool is regulated by efficiency of intestinal absorption and hepatic biosynthesis.

Cyclosporin A (CsA), a drug that has successfully been applied for immunosuppression after solid organ transplantation, is associated with a number of side effects, including nephrotoxicity, hyperlipidemia, and hepatotoxicity. Cholestasis and cholelithiasis, i.e., disturbances of bile formation, have repeatedly been reported in patients on CsA therapy. CsA has been shown to interact with various steps of bile salt metabolism. CsA acutely inhibits bile salt synthesis in cultured rat and human hepatocytes. Reductions of bile salt synthesis and bile flow have been reported in rats treated with CsA. CsA also interferes with hepatocytic bile salt transport. The drug competitively inhibits sodium-dependent uptake of radiolabeled taurocholate by rat hepatocytes and by liver plasma membrane vesicles. In canalicular liver plasma membrane vesicles, CsA impairs ATP-dependent transport of taurocholate. Hepatobiliary secretion of intravenously administered radiolabeled taurocholate was inhibited in bile fistula rats that were acutely or chronically treated with CsA. Finally, CsA may also directly affect bile salt handling by the intestine: ileal perfusion with CsA was shown to impair intestinal bile salt absorption in rats.

Thus, the effects of CsA on separate processes involved in the enterohepatic cycling of bile salts have been studied rather extensively, predominantly in in vitro systems and in vivo after surgical interruption of the enterohepatic circulation in animals. However, no integrated in vivo data are available on the effects of chronic CsA treatment. To obtain an integrated view of the effects of CsA on the enterohepatic circulation of bile salts, we studied parameters of the enterohepatic circulation of cholate, a quantitatively major bile salt species in the rat, in relation to the expression of transport proteins involved in the enterohepatic circulation (i.e., Ntcp, Oatp1, Bsep, and Asbt). Gene expression of ileal bile salt binding protein (Ilbp), a cytosolic protein implicated in control of intestinal bile salt reabsorption, and expression of the nuclear bile salt receptor FXR, known to be involved in control of Ilbp expression, as well as gene expression of the truncated form of Asbt (tAsbt), a candidate protein for basolateral bile salt transport, were also assessed. A recently developed stable isotope dilution method was used for
quantification of cholate kinetics in unanesthetized rats\textsuperscript{15}.

The results demonstrate that CsA inhibits the synthesis of cholate, but does not affect its pool size. Maintenance of the cholate pool size is associated with more efficient absorption of cholate from the intestinal lumen and with increased Asbt protein expression in the distal ileum of CsA-treated rats.

**MATERIAL AND METHODS**

**Animals**

Male Wistar rats (mean body weight $\pm$ S.D., 339 $\pm$ 29 g; Harlan Laboratories, Zeist, The Netherlands) were kept in a light- and temperature-controlled environment. They were fed standard rodent diet (RMH-B; Hope Farms BV, Woerden, The Netherlands) and tap water ad libitum. Experimental protocols were approved by the local Ethical Committee for Animal Experiments.

**Materials**

$[2,2,4,4-\text{H}_4]$-Cholate ($[\text{H}_4]$-cholate, isotopic purity 98\%) was obtained from Isotech Inc. (Miamisburg, OH). Cholylglycine hydrolase from *Clostridium perfringens* (*welchii*) was purchased from Sigma-Aldrich (St. Louis, MO). Pentafluorobenzylbromide was purchased from Fluka Chemie (Buchs, Neu-Ulm, Switzerland). All other chemicals and solvents used were of the highest purity commercially available.

**Experimental Procedures**

Rats were equipped with a permanent heart catheter under halothane anesthesia as described previously\textsuperscript{16}. After recovery for 1 week, rats were injected daily subcutaneously with CsA (dose 1 mg·100g$^{-1}$·day$^{-1}$) or with its vehiculum [Cremophor EL, 650 mg/ml and ethanol 33\% (v/v)] for 10 days. At day 7, $[\text{H}_4]$-cholate (dose 5 mg/rat) was intravenously administered to CsA-treated and control rats. Subsequently, blood samples (0.25 mL) were obtained between days 7 and 10 at 1, 3, 6, 9, 12, 21, 28, and 48 h after administration of $[\text{H}_4]$-cholate. Plasma was obtained by centrifugation at 4000 rpm for 10 min and stored at 20°C until analysis. At day 10, animals were anesthetized by intraperitoneal injection of sodium pentobarbital and, after collection of a single 15-min bile sample via a bile fistula, the liver and small intestine of the animals were quickly removed. The 30-cm distal end of the small intestine was rinsed with 10 ml of 1 mM NaHCO$_3$ buffer (pH 7.4) containing phenylmethylsulfonylfluoride to prevent protein degradation and divided into proximal, mid-, and distal segments of 10 cm. After collection, tissue samples were immediately frozen in liquid nitrogen and stored at 80°C for membrane preparation and RNA isolation.

**Analytical Procedures**

Plasma alanine transaminase, aspartate transaminase, alkaline phosphatase, bilirubin, cholesterol, and triglycerides were determined by routine laboratory techniques. Total bile salts in plasma, bile, and feces were determined by an enzymatic fluorometric assay using 3$\alpha$-hydroxysteroid dehydrogenase\textsuperscript{17}. Whole blood concentrations of CsA were determined by use of an enzymatic multiplied
immunoassay technique\textsuperscript{18}.

**Gas-Liquid Chromatography Electron Capture Negative Chemical Ionization Mass Spectrometry**

Plasma samples were prepared for bile salt analysis by gas chromatography-mass spectrometry\textsuperscript{15}. All analyses were performed on an SSQ7000 quadrupole gas chromatography-mass spectrometry instrument (Thermo Finnigan, San Jose, CA). GC separation was performed on a 30 m × 0.25 mm column, 0.25-µm film thickness (DB-5MS; J&W Scientific, Folsom, CA).

**Gas Chromatography**

Bile salt composition of bile and feces samples were determined by capillary gas chromatography on a Hewlett Packard gas chromatograph (HP 5880A), equipped with a 50 m × 0.32 mm CP-Sil-19 fused silica column (Chrompack BV, Middelburg, The Netherlands). For this purpose, bile salts were converted to methylester-trimethylsilyl derivatives. Quantification of bile salts was performed by adding coprostanol as internal standard.

**Preparation of Hepatic and Intestinal Membranes for Protein Analysis**

Isolation of hepatic plasma membranes was performed as described previously\textsuperscript{19}. Intestinal brush-border membranes were isolated as described by Schmitz et al.\textsuperscript{20}. Total protein concentration of membrane fractions was determined using the method described by Lowry et al.\textsuperscript{21}. To determine the degree of purification of the isolated membrane fractions, activities of marker enzymes in the membrane fractions were divided by activities in the corresponding homogenates. Na\textsuperscript{+}/K\textsuperscript{+}-ATPase and Mg\textsuperscript{2+}-ATPase were used for the basolateral and canalicular fractions of liver plasma membranes, respectively\textsuperscript{22}. For the intestinal brush-border membranes alkaline phosphatase was used as a marker enzyme\textsuperscript{23}. The amounts of protein of liver plasma membrane fractions and intestinal brush-border fractions used for gel electrophoresis were standardized to achieve similar relative enrichments of the respective marker enzymes. Separation of proteins was performed on 4 to 15% gradient gels (Bio-Rad, Hercules, CA) and proteins were transferred to ECL-Hybond nitrocellulose (Amersham Biosciences UK, Ltd., Little Chalfont, Buckinghamshire, UK) by Western blotting. Liver samples were probed with anti-Ntcp-immunoglobulin(Ig)G K4, anti-Oatp1-immunoglobulin IgG K10 [gift from Prof. Dr. P. J. Meier-Abt and Dr. B. Stieger (Division of Clinical Pharmacology and Toxicology, Department of Medicine, University, Hospital, Zürich, Switzerland)]\textsuperscript{24}, anti-Bsep IgG\textsuperscript{1}, or anti-Mrp2 IgG (both kindly provided by Prof. Dr. M. Müller, University of Wageningen, Wageningen, The Netherlands)\textsuperscript{25}, respectively. Asbt protein content of brush-border membranes was determined using polyclonal anti-rat Asbt antibody\textsuperscript{26}. Detection of immune complexes in liver and intestinal membranes was performed using anti-rabbit or anti-guinea pig antibody, respectively, linked to horseradish peroxidase (Sigma-Aldrich) as secondary antibody and enhanced chemiluminescence as provided by the manufacturers (Amersham Biosciences UK, Ltd.). Intensities of the protein bands were measured by densitometry and relative amounts compared with controls were determined.
RNA Isolation and PCR Procedures
Total RNA from the three intestinal sections per animal was isolated and reverse transcribed as described previously\textsuperscript{27}. Real-time quantitative PCR was performed using a 7700 sequence detector according to the manufacturer’s instructions (Applied Biosystems, Foster City, CA). Primers were obtained from Invitrogen (Carlsbad, CA). Fluorogenic probes, labeled with 6-carboxy-fluorescein and 6-carboxy-tetramethyl-rhodamine, were made by Eurogentec (Seraing, Belgium). Primers and probes were as follows: \(\beta\)-actin (NM\_031144, sense AGCCATGTACGTAGCCATCCA, antisense TCTCCGTAGTATGCCTCTGTCGATACCTCAGATG, and probe TGTCCCTGTATGCCTCTGTCGATACCTCAGATG), FXR (U18374, sense CGCTGAGATGGATATGTCTTG, antisense CCTTCACTGCACATCCCAGAT, and probe TGACCACAAGTTCAACCCGCTCTC); Ilbp (NM\_008375, sense CCCCACTATCACCCAGACTCG, antisense ACATCCCCGATGGAGATAG, and probe TCCACAACTTTGCAAGAATGGC); Asbt (U07183, sense ACCACTTGCTCCAGACTGCTT, antisense CGTTCCTGAGTCAACCCGCTCTC, and probe CTGGGAATGATGCCCGCTTGCCTCT); and truncated Asbt (U07183, sense AGGCTGTGGTGGAGATAG, antisense CAGAGAGGCTAGACTGCTT, and probe CTCGCCCTGAGAATGGCCCGCTCTCAA). Asbt primers are situated in exon 2, which is skipped in tAsbt; the antisense tAsbt primer consists of two halves, one in exon 1, the other in exon 3. Therefore, this PCR setup can differentiate between the functionally different Asbt and tAsbt. All expression data were subsequently standardized for \(\beta\)-actin, which was analyzed in separate runs. Glutathione analysis in bile was performed as described previously\textsuperscript{28}.

Calculations

**Isotope Dilution Technique.**
The isotope dilution technique was performed as described by Hulzebos et al.\textsuperscript{15}. Enrichment was defined as the increase of \((M_4-CA)/M_0-CA\) relative to baseline measurements after administration of \([\text{H}_4]\)-CA and expressed as the natural logarithm of atom percent excess (\(\ln\ APE\)) value\textsuperscript{29}. 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 (per day) equals the slope of the regression line. The pool size (micromoles per 100 grams) is determined according to the formula \((D \cdot b \cdot 100)/a\) - \(D\), 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 (micromoles per 100 grams per day) was determined by multiplying pool size and FTR.

**Cycling Time.**
The cholate cycling time, \textit{i.e.}, the time it takes the cholate pool to circulate one time in the enterohepatic circulation, was calculated by dividing the cholate pool size (micromoles per 100 grams) by the biliary secretion rate of cholate (micromoles per hour per 100 grams). The cholate biliary secretion rate was calculated by multiplying the bile flow (microliters per hour per 100 grams) with the cholate concentration (millimolar) in a single 15-min fraction, obtained immediately after cannulation of the common bile duct. The fraction of cholate lost
per enterohepatic cycle was subsequently calculated by dividing fractional cholate synthesis rate by cholate cycling frequency and was expressed as percentage of total cholate pool size (%CA 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 CsA-treated and control rats were evaluated by Rank test or Mann-Whitney exact two-tailed *U* test. Level of significance for all statistical analyses was set at *p* < 0.05. Analysis was performed using SPSS 8.5 for Windows software (SPSS, Chicago, IL).

RESULTS

Animal Characteristics and Effects of Chronic CsA Treatment on Parameters of Liver Function.
Body weights of CsA-treated animals were decreased compared with those of controls (Table 1), mainly due to weight loss during the first 5 days after onset of CsA treatment. The liver weight/body weight ratio was unaffected by CsA treatment. CsA treatment was associated with elevated plasma bile salt and bilirubin concentrations, whereas alanine transaminase activity was slightly decreased (Table 1). There were no differences in alkaline phoshatase and aspartate transaminase activities nor in plasma cholesterol and triglyceride concentrations between CsA-treated and control rats. The CsA levels after 10 days of treatment were 3.9 ± 1.2 mg/l. CsA treatment was associated with a 31% reduction of bile flow rate after acute interruption of the enterohepatic circulation (Table 2). Total biliary bile salt secretion rate as well as the biliary cholate secretion, as measured during the first 15 min after initiation of bile

**TABLE 1.** Animal characteristics and plasma parameters of liver function

<table>
<thead>
<tr>
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<th>Control</th>
<th>CsA</th>
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<tbody>
<tr>
<td>Body weight (g)</td>
<td>364 ± 9</td>
<td>313 ± 11***</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>12.8 ± 0.4</td>
<td>11.4 ± 1.2</td>
</tr>
<tr>
<td>Liver/body weight ratio</td>
<td>0.035 ± 0.001</td>
<td>0.036 ± 0.003</td>
</tr>
<tr>
<td>Bile salts (µmol/l)</td>
<td>44.2 ± 16.6</td>
<td>180.6 ± 32.3***</td>
</tr>
<tr>
<td>Total bilirubin (µmol/l)</td>
<td>8.2 ± 0.8</td>
<td>12.0 ± 1.2*</td>
</tr>
<tr>
<td>Alanine transaminase (units/l)</td>
<td>41.6 ± 2.3</td>
<td>28.8 ± 6.6*</td>
</tr>
<tr>
<td>Aspartate transaminase (units/l)</td>
<td>98.6 ± 25.1</td>
<td>69.8 ± 15.6</td>
</tr>
<tr>
<td>Alkaline phosphatase (units/l)</td>
<td>127.2 ± 17.2</td>
<td>121.4 ± 31.1</td>
</tr>
<tr>
<td>Cholesterol (mmol/l)</td>
<td>1.4 ± 0.2</td>
<td>1.5 ± 0.2</td>
</tr>
<tr>
<td>Triglycerides (mmol/l)</td>
<td>1.0 ± 0.6</td>
<td>0.9 ± 0.3</td>
</tr>
<tr>
<td>Cyclosporin (mg/l)</td>
<td>&lt;0.025</td>
<td>3.9 ± 1.2***</td>
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</table>

CsA vehiculum (Cremophor EL, controls) or CsA alone were given for 1 week. CsA (s.c.) was administered at doses of 1 mg·100g⁻¹·day⁻¹. Data are expressed as means ± S.D. of control and CsA-treated rats. *** *p* < 0.001; * *p* < 0.05; n = 5 rats/group.
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**Figure 1.** Decay of intravenously administered $[^{2}H_4]$-cholate (5 mg/rat) in control (open circles) and CsA-treated (closed circles) rats. Data are means ± standard deviation of $n = 4$ rats/group.

**Figure 2.** Effects of CsA treatment on FTR (**A**), pool size (**B**), synthesis rate (**C**), and calculated fecal loss of cholate (**D**) in control (open squares) and CsA-treated (closed squares) rats. Data are means ± SD of $n = 4$ rats/group. CsA-treated rats significantly different from controls, *, $p < 0.05$; ***, $p < 0.001$.

**TABLE 2.** Effects of chronic CsA treatment on bile formation

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>CsA</th>
</tr>
</thead>
<tbody>
<tr>
<td>Bile flow ($\mu l \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$)</td>
<td>5.5 ± 0.5</td>
<td>3.8 ± 0.6*</td>
</tr>
<tr>
<td>Bile salt secretion rate ($\text{nmol} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$)</td>
<td>270 ± 54</td>
<td>264 ± 35</td>
</tr>
<tr>
<td>Cholate secretion rate ($\text{nmol} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$)</td>
<td>177 ± 28</td>
<td>165 ± 15</td>
</tr>
<tr>
<td>Glutathione secretion rate ($\text{nmol} \cdot 100 \text{ g}^{-1} \cdot \text{min}^{-1}$)</td>
<td>22.7 ± 4.8</td>
<td>11.2 ± 5.9*</td>
</tr>
</tbody>
</table>

CsA vehiculum (Cremophor EL, controls) or CsA alone were given for 1 week. CsA (s.c.) was administered at doses of 1 mg $\cdot 100 \text{ g}^{-1} \cdot \text{day}^{-1}$. Data are expressed as means ± SD of control and CsA-treated rats. * $p < 0.05$; $n = 5$ rats/group.
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collection were not significantly altered in the CsA-treated animals. In contrast, glutathione secretion into bile was significantly lower in the CsA-treated rats.

**Effects of Chronic CsA Treatment on Kinetic Parameters of Cholate Metabolism.**
Analysis of plasma cholate enrichments over time indicated that deuterated cholate disappeared from plasma at a slower rate in the CsA-treated rats than in controls (Fig. 1). The fractional turnover rate of cholate (Fig. 2A), calculated from the absolute value of the slope of the linear regression curve (Fig. 1), was reduced by 65% in CsA-treated rats compared with controls (0.25 ± 0.07 versus 0.72 ± 0.14 pools · day⁻¹, respectively; p < 0.001). The cholate pool size (Fig. 2B), calculated from the y-intercept of the linear regression line (Fig. 1), was similar in both groups (12.7 ± 2.2 versus 15.2 ± 2.3 µmol·100 g⁻¹, CsA-treated rats versus controls; N.S.). Total biliary bile salt concentration was not similar in both groups (70.7 ± 7.8 versus 49.4 ± 7.3 mM, CsA-treated rats versus controls; p < 0.05). Quantitatively, the cholate fraction accounted for more than 60% of the biliary bile salts (63.3 ± 7.8 versus 66.1 ± 3.5%, CsA-treated rats versus controls; N.S.). Under the assumption that the different bile salt species displayed a similar cycling frequency, the calculated total pool sizes of non-cholate bile salts were 7.9 ± 4.4 µmol · 100 g⁻¹ after CsA treatment and 7.8 ± 1.2 µmol·100 g⁻¹ in control rats (N.S.).

CsA decreased the cholate synthesis rate (Fig. 2C) by 71% compared with controls (3.2 ± 1.1 versus 10.8 ± 1.6 µmol·100 g⁻¹·day⁻¹, CsA-treated rats versus controls; p < 0.001). In accordance with the reduced cholate synthesis rate determined by stable isotope dilution in a steady-state condition, CsA decreased fecal loss of bile salts by ~30% (4.2 ± 1.4 versus 6.1 ± 0.5 µmol·100·g⁻¹·day⁻¹, CsA-treated versus control rats; p < 0.05).

The cholate cycling time was not affected by CsA treatment (1.3 ± 0.3 versus 1.5 ± 0.3 h, CsA-treated versus control rats; N.S.). Consequently, CsA reduced the calculated percentage of cholate lost per enterohepatic cycle (Fig. 2D) by ~70% (1.4 ± 0.5 versus 4.3 ± 0.5% CA pool size, CsA-treated versus control rats; p < 0.001).

**Effects of Chronic CsA Treatment on Hepatic Transporters (Ntcp, Oatp1, Bsep, and Mrp2).**
CsA treatment did not significantly affect protein levels of Ntcp, Oatp1, Bsep, and of Mrp2 in isolated hepatic plasma membrane fractions, although the latter, on average, was slightly reduced (Fig. 3).

**Intestinal mRNA Expression Patterns of Bile Salt Transporters.**
CsA treatment did not affect mRNA expression levels of genes encoding transport proteins putatively involved in bile salt uptake (Asbt), basolateral efflux (tAsbt), and intracellular trafficking (Ilbp) (Fig. 4). The mRNA levels of genes encoding these proteins, which were measured in different sections derived from the 30-cm distal end of small intestine, were almost absent in the proximal segment, and showed a steep increase toward the distal ileum. A similar expression pattern was observed for the nuclear bile salt receptor FXR.
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Figure 3. Protein levels of Ntcp, Oatp1, Bsep, and Mrp2 in hepatic plasma membrane fractions isolated from CsA-treated and control rats; $n = 5$/group. Analysis was done as described under Materials and Methods. The relative amounts of Ntcp, Oatp1, Bsep, and Mrp2 were not significantly affected by CsA treatment compared with controls (100%) (Ntcp, 210 ± 90%; Oatp1, 90 ± 40%; Bsep, 150 ± 30%; and Mrp2, 70 ± 30%).

Figure 4. Intestinal mRNA expression patterns of bile salt transporters and a nuclear bile salt receptor (FXR) in the small intestine of control (open circles) and CsA-treated (closed circles) rats. Analysis was done as described under Materials and Methods. All expression data were standardized for β-actin. Results are shown for three sequential sections of the 30-cm distal end of the small intestine, i.e., proximal, mid, and distal ileum. CsA treatment did not affect gene expression levels of transport proteins involved in bile salt uptake (Asbt), basolateral efflux (tAsbt), intracellular trafficking (Ilbp), or the level of FXR. Data are means ± standard deviation of $n = 5$ rats/group.

Asbt Protein Levels in Distal Segments of Small Intestine.
Immunoblotting experiments with brush-border membrane fractions from different intestinal sections derived from the terminal 30 cm of the ileum of from CsA-treated and control rats demonstrated that expression of Asbt protein was mainly confined to the most distal segments in control rats and shifted toward more proximal segments after CsA treatment (Fig.5, top). Asbt protein content was approximately 2-fold increased in CsA-treated rats compared with controls in the most distal part of the small intestine (Fig.5, bottom).
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Figure 5. Asbt protein levels in the 30-cm distal end of the small intestine, i.e., proximal (1), mid (2), and distal (3) ileum, of control and CsA-treated rats by Western blot analysis (top). Analysis was done as described under Materials and Methods. The amounts of protein loaded onto the gel were standardized to similar activities of alkaline phosphatase. Results are shown for three sequential different distal brush-border membrane fractions per animal, and for two animals per group that are representative for n = 4/group. Quantification of Asbt protein expression in distal (3) ileum after CsA treatment (bottom). Immunoreactive bands, detected by Western blot analysis (top) were evaluated with densitometry. Asbt levels were expressed as percentage of the control group. Data are means ± standard deviation of n = 4 rats/group, expressed as percentage of the control group. CsA-treated rats significantly different from controls, *, p < 0.05.

Figure 6. Effects of CsA on the enterohepatic circulation of CA. CA synthesis rate is markedly decreased after CsA treatment, whereas biliary CA secretion rate and CA cycling time are unaffected. CsA reduced the calculated percentage of CA lost per enterohepatic cycle in association with an increased intestinal Asbt protein (closed circles).
This present study is the first to describe the effects of CsA on the enterohepatic circulation of bile salts in unanesthetized rats in vivo. In accordance with available in vitro data, it was confirmed that CsA profoundly inhibits the synthesis of the primary bile salt cholate in vivo. Yet, the size of cholate pool undergoing enterohepatic circulation is not reduced, but rather maintained through more efficient absorption of cholate from the intestine: both the calculated percentage of cholate lost per enterohepatic cycle and the amount of bile salts lost in the feces were markedly lower in CsA-treated rats than in controls. We speculate that induction of Asbt expression in the distal ileum contributes to more efficient absorption of cholate from the intestinal lumen and is responsible for the maintenance of the cholate pool size during CsA treatment (summarized in Fig.6).

CsA is known to interfere with hepatobiliary bile salt transport. Accordingly, CsA-treated rats showed elevated plasma bile salt levels. However, CsA treatment does not influence biliary bile salt secretion rate, which demonstrates that interference of CsA with hepatic transporters does not affect net hepatobiliary transport rates in steady-state conditions, albeit at increased serum bile salt concentrations. Elevated plasma bile salts in CsA-treated rats may result from interference of CsA with hepatic uptake, transcellular transport, and/or canalicular secretion of bile salts. After CsA treatment, however, no significant changes in protein expression of Ntcp, Oatp1 (bile salt uptake), and Bsep (bile salt secretion) were observed. It thus seems more likely that CsA treatment interferes directly with these bile salt transporting systems, for example, by competitive inhibition as reported previously. Also, CsA has been demonstrated to interfere directly with Mrp2-mediated transport activity. Moreover, CsA alters liver plasma membrane composition, fluidity, and depletes hepatic glutathione content. In agreement with this in vitro observation, we found biliary glutathione secretion rate to be markedly diminished after CsA treatment in the presence of only slightly reduced levels of Mrp2 protein, the canalicular transporter responsible for biliary glutathione secretion. Biliary secretion of glutathione significantly contributes to generation of the bile salt-independent fraction of bile flow. Therefore, the reduction of bile flow in the CsA-treated group is probably mainly caused by reduction of glutathione secretion.

CsA reduced cholate synthesis by 70%. Yet, hepatic mRNA levels of both Cyp7A1 and Cyp27 were increased by ∼300 and ∼150%, respectively, in CsA-treated rats (data not shown). Impaired cholate synthesis could thus be related to interference of CsA with enzyme activities or to CsA-induced changes in the relevant precursor pool sizes. Whether the drug similarly affects only the activities of Cyp7A1 and Cyp27 or also activities of enzymes further downstream in the cascades of the acidic and neutral pathway of bile salt synthesis cannot be deduced from the data presented. The latter option is likely in view of the largely unchanged bile salt pool composition after CsA treatment.

For the genes involved in intestinal bile salt transport (i.e., Asbt, Ilbp, and tAsbt) and one of the key regulators, i.e., the bile salt receptor FXR, we found highest expression in the distal segment of the terminal ileum of rats, in agreement with reported data. Yet, no significant differences were found between
CsA-treated and control rats.

The profound inhibitory effects of CsA on bile salt synthesis in vivo are in line with previous observations in cultured rat and human hepatocytes. In a previous in vivo rat study, a daily treatment with CsA for 1 week led to ~50% reduction of total bile salt synthesis, as determined by the washout technique applied to anesthetized animals. In the present study, we have focused on the kinetics of cholate metabolism using a novel microscale isotope dilution technique, applicable in unanesthetized animals. In contrast to previous data on CsA treatment in rats, obtained by the washout technique, we did not observe a significant change in cholate pool size or in the (calculated) total bile salt pool size. Differences in experimental setup, i.e., use of unanesthetized animals with intact enterohepatic circulation, different strains or ages of rats, or the use of different methods may contribute to this discrepancy. It should be realized that introduction of an acute bile fistula after anesthesia is not without possible artifact on bile salt output and the use of an unanesthetized rat model with exteriorized enterohepatic circulation would be most optimal. However, it is anticipated that potentially interfering effects have been similar in both groups studied. In previous studies, it was hypothesized that reduced bile salt synthesis contributes to the concomitantly observed reduction in bile salt pool size. Our data clearly demonstrate that decreased bile salt synthesis does not necessarily lead to a reduced bile salt pool size. Rather, our data provide three indications that the lower bile salt synthesis is compensated for by a more efficient intestinal bile salt conservation during chronic CsA treatment.

First, we were able to calculate the fraction of the cholate pool that escapes intestinal absorption per enterohepatic cycle; cholate constitutes quantitatively the major fraction of the bile salt pool in rats and CsA induces only minor changes in biliary bile salt composition. The time needed for a cholate molecule to undergo one full enterohepatic cycle, the “cycling time”, was determined in control and CsA-treated rats. During CsA treatment, the calculated percentage of cholate lost per enterohepatic cycle was ~70% lower than in control rats. Second, the strongly decreased fecal bile salt excretion rate in the face of unchanged rate of bile salt secretion in bile, cycling time, and pool size also supports more effective intestinal conservation of bile salts during CsA treatment. Third, the increased expression of Asbt in the intestinal mucosa of CsA-treated rats favors the possibility that intestinal bile salt absorption efficiency is enhanced at this level. A possible role of Asbt in intrahepatic bile duct cells in cyclosporin A-induced changes of cholate kinetics seems unlikely; Asbt expression in crude plasma membranes of total liver was not significantly affected by CsA (data not shown).

The present data thus indicate that, despite a profound inhibition of bile salt biosynthesis, the bile salt pool size is maintained in CsA-treated rats by a more efficient intestinal absorption. The “classic view” implies that bile salt pool size is maintained by bile salt synthesis, which, under steady-state conditions, compensates for fecal bile salt loss. This view is supported by the frequently observed increase in bile salt synthesis in rodents and in humans after ingestion of cholestyramine, a bile salt-binding resin that enhances fecal bile salt excretion. The increased bile salt synthesis during cholestyramine treatment is mediated by alleviation of feedback repression of synthesis through the action of FXR, a
nuclear receptor that is activated by bile salts\textsuperscript{2}. Various studies\textsuperscript{34-35}, including the present one, however, indicate that regulation of the bile salt pool size may not only occur at the level of hepatic biosynthesis in response to intestinal events. Rather, data strongly suggest that intestinal events can influence the bile salt pool size independently. Lillienau \textit{et al.}\textsuperscript{36} found functional ileal bile salt transport to be up-regulated by cholestyramine and down-regulated by glycocholate feeding, each of which may serve to maintain a constant bile salt pool size. Yet, the regulation of ileal bile salt transport has not been fully characterized yet, and seemingly conflicting reports on up- or down-regulation by intestinal bile salts have been published\textsuperscript{37-38}. In our study, the influx of bile salts into the intestine was not altered in CsA-treated animals, whereas the total amount of Asbt protein in the terminal ileum was clearly increased. It seems therefore possible that another factor than the intestinal bile salt flux, either directly or indirectly, influences ileal bile salt transport by altering Asbt protein levels. The discrepancy between Asbt mRNA levels and Asbt protein expression indicates post-transcriptional events to be involved, \textit{e.g.}, stabilization of the protein. The induction of Asbt may be a direct consequence of CsA treatment, for instance, related to CsA-induced intestinal hemodynamic and functional impairment\textsuperscript{39}. It cannot be ruled out, however, that CsA mediates its effects on bile salt reabsorption by indirect means, \textit{e.g.}, by effects on intestinal motility\textsuperscript{40}.

In conclusion, CsA inhibits bile salt synthesis, without affecting bile salt pool size or the enterohepatic cycling time of cholate in rats. The calculated percentage cholate that is lost per enterohepatic cycle as well as the total fecal bile salt loss are reduced. We speculate that the concomitantly observed increase in Asbt protein expression in CsA-treated rats is involved in a more efficient intestinal bile salt absorption and exerts a regulatory role in maintenance of the bile salt pool size.

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