Fxr$^{-/-}$ mice adapt to biliary obstruction by enhanced phase I detoxification and renal elimination of bile acids

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Abstract **Farnesoid X receptor knockout (*Fxr*$^{-/-}$) mice cannot upregulate the bile salt export pump in bile acid loading or cholestatic conditions. To investigate whether *Fxr*$^{-/-}$ mice differ in bile acid detoxification compared with wild-type mice, we performed a comprehensive analysis of bile acids extracted from liver, bile, serum, and urine of naive and common bile duct-ligated wild-type and *Fxr*$^{-/-}$ mice using electrospray and gas chromatography mass spectrometry. In addition, hepatic and renal gene expression levels of *Cyp2b10* and *Cyp3a11*, and protein expression levels of putative renal bile acid-transporting proteins, were investigated. We found significantly enhanced hepatic bile acid hydroxylation in *Fxr*$^{-/-}$ mice, in particular hydroxylations of cholic acid in the 1β, 2β, 4β, 6α, 6β, 22, or 23 position and a significantly enhanced excretion of these metabolites in urine. The gene expression level of Cyp3a11 was increased in the liver of *Fxr*$^{-/-}$ mice, whereas the protein expression levels of multidrug resistance-related protein 4 (Mrp4) were increased in kidneys of both genotypes during common bile duct ligation. In conclusion, *Fxr*$^{-/-}$ mice detoxify accumulating bile acids in the liver by enhanced hydroxylation reactions probably catalyzed by Cyp3a11. The metabolites formed were excreted into urine, most likely with the participation of Mrp4.**—Marschall, H-U., M. Wagner, K. Bodin, G. Zollner, P. Fickert, J. Gumhold, D. Silbert, A. Fuchsbichler, J. Sjövall, and M. Trauner. ***Fxr*$^{-/-}$ **mice adapt to biliary obstruction by enhanced phase I detoxification and renal elimination of bile acids. *J. Lipid Res.* 2006.** 47: **582–592.**

Supplementary key words farnesoid X receptor knockout • multidrug resistance-related protein 4 • cytochrome 3a11 • gas chromatography-mass spectrometry • electrospray mass spectrometry

The nuclear farnesoid X receptor (FXR) mediates bile acid effects on the expression of various genes involved in bile acid metabolism and transport (1–3). The central role of FXR in bile acid homeostasis has been established in mice with targeted disruption of *Fxr* (*Fxr*$^{-/-}$ mice) under conditions of bile acid loading [i.e., cholic acid (CA) or ursodeoxycholic acid (UDCA) feeding (4–6) and common bile duct ligation (CBDL) (7)]. When studying the adaptive response of hepatic ABC transport proteins, we found that CBDL induced the expression of multidrug resistance-related protein 3 (Mrp3) and Mrp4 in wild-type mice and even more in *Fxr*$^{-/-}$ mice, whereas Mrp2 expression remained unchanged (7). FXR-independent induction of hepatic Mrp2–Mrp4 as well as of renal Mrp2 and Mrp4 expression was also seen during CA and UDCA feeding (5, 8). In contrast, a striking FXR dependence was seen in the regulation of the bile salt export pump (Bsep), because *Fxr*$^{-/-}$ mice failed to upregulate Bsep in any bile acid-loading condition (7). Nevertheless, alanine aminotransferase levels and mortality rates did not differ between wild-type and *Fxr*$^{-/-}$ mice in obstructive cholestasis (7). Rather, *Fxr*$^{-/-}$ mice had significantly lower levels of bile acids in the liver tissue and serum than did wild-type animals. This was even more surprising in light of the inability of *Fxr*$^{-/-}$ mice to decrease the level of hepatotoxic CA (4, 5) in the liver via downregulation of *Cyp7a1* and *Cyp8b1*, as shown previously (7).

This study thus aims to determine *i*) whether differences in the bile acid profiles may account for a more efficient elimination in *Fxr*$^{-/-}$ mice than in their wild-type relatives, *ii*) whether these compounds are formed by enhanced phase I or phase II detoxification reactions, and *iii*) whether changes in renal bile acid transporter expression also contribute to increased bile acid elimination.

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Abbreviations: Asbt, apical sodium-dependent bile acid transporter; Bsep, bile salt export pump; CA, cholic acid; CBDL, common bile duct ligation; DCA, deoxycholic acid; ES-MS, electrospray mass spectrometry; FXR, farnesoid X receptor; LCA, lithocholic acid; MCA, muricholic acid; MeTMS, methyl ester trimethylsilyl ether; Mrp, multidrug resistance-related protein; Oatp1, organic anion-transporting polypeptide 1; PXR, pregnane X receptor; RI, retention index; UDCA, ursodeoxycholic acid.

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EXPERIMENTAL PROCEDURES

Animals

C57/BL6 mice, 25–30 g, with targeted disruption of *Fxr* (4), obtained from Frank J. Gonzalez (National Cancer Institute, National Institutes of Health, Bethesda, MD), and wild-type littermates were housed with a 12/12 h light/dark cycle and permitted ad libitum consumption of water and a standard mouse diet. The experimental protocol was approved by the local Animal Care and Use Committee, according to criteria outlined in the "Guide for the Care and Use of Laboratory Animals" prepared by the National Academy of Sciences (National Institutes of Health publication 86-23, revised 1985).

CBDL

To study the role of FXR in changes in bile acid metabolism and the expression of renal bile acid transporters in cholestasis, 2 month old male wild-type and $Fxr^{-/-}$ mice were subjected to bile duct ligation and cholecystectomy, as described previously (7). Sham-operated animals were subjected to the same surgical procedure, but without ligation of the common bile duct and removal of the gallbladder. Livers were excised under general anesthesia with avertin (400 mg/kg body weight, intraperitoneally) 3 and 7 days after surgery, respectively (three to five animals were studied in each group). Urine was collected in metabolic cages, and bile was sampled by puncturing of the gallbladder in naive mice or the dilated bile duct after CBDL.

Bile acid measurements

Bile acids were extracted from liver homogenates with 80% methanol according to Setchell et al. (9). From bile (~0.05 ml/animal), serum (~0.1 ml/animal), and urine (2–5 ml/animal/24 h), bile acids were extracted using solid-phase extraction as described (10), in the case of bile and serum after disrupting protein adsorption by incubating with 1 ml of 0.5 M NEt_3HSO_4 at 64°C for 30 min. Equipment and conditions used for electrospray mass spectrometry (ES-MS) and sample purification by anion-exchange chromatography, hydrolysis by cholylglycine hydrolase, and conversion to methyl ester trimethylsilyl ether (MeTMS) derivatives for GC-MS were the same as described previously in detail for the quantification of bile acids in individual human serum and urine samples (10). For GC-MS, 1 μl of each sample was injected in splitless mode. The compounds were separated on a HP-1 column with the following temperature program: hold at 180°C for 1 min, increase from 180 to 220°C at a rate of 20°C/min, and finally increase from 220 to 290°C at 3.5°C/min. A full-scan spectrum (*m/z* 100–800) was recorded for each compound. Reference primary and secondary bile acids were obtained from Sigma-Aldrich (St. Louis, MO). Reference 1β-, 2β-, 4β-, or 6α-hydroxylated CA (3α,7α,12α-trihydroxy-5β-cholan-24-oic acid) and 12α-hydroxy-β-muricholic acid (β-MCA; 3α,6β,7β-trihydroxy-5β-cholan-24-oic acid) were kind gifts of Prof. Takashi Iida. Further identification of polyhydroxylated bile acids was possible by comparison with published spectra of bile acid MeTMS derivatives (11, 12). Retention indices (RIs) as given in **Tables 1–3** relate to the elution of normal

TABLE 1. Liver tissue bile acids in wild-type and $Fxr^{-/-}$ mice during biliary obstruction

Bile Acids	RI	Naive		3 d CBDL		7 d CDBL	
		Wild Type	$Fxr^{-/-}$	Wild Type	$Fxr^{-/-}$	Wild Type	$Fxr^{-/-}$
		% total					
Total (μmol/g)		0.07 ± 0.04	0.10 ± 0.07	1.00 ± 0.27[a]	0.79 ± 0.47[a]	0.92 ± 0.43[a]	0.36 ± 0.16[a,b]
Primary and secondary							
LCA	3107	3.5 ± 3.0	1.2 ± 1.4	0.1 ± 0.1	1.1 ± 1.4	0.1 ± 0.1	0.3 ± 0.4
allo-CA	3173			0.5 ± 0.4		0.4 ± 0.4	
DCA	3177	15.3 ± 10.5	25.6 ± 8.8	1.4 ± 1.6	2.2 ± 3.1	0.4 ± 0.5	0.7 ± 0.7
CDCA	3195	1.2 ± 1.1	1.7 ± 0.5	0.3 ± 0.1	0.4 ± 0.3	0.3 ± 0.3	0.5 ± 0.5
α-MCA	3200	4.5 ± 2.8	1.7 ± 1.4	0.7 ± 0.4	0.9 ± 0.6	0.8 ± 0.4	0.8 ± 0.4
CA	3214	42.3 ± 8.8	44.0 ± 11.3	43.4 ± 16.6	49.9 ± 25.1	11.9 ± 1.0	41.1 ± 19.3
UDCA	3230	2.9 ± 3.1	0.7 ± 0.8	1.0 ± 0.6	1.2 ± 1.0	1.0 ± 0.6	0.4 ± 0.3
HCA	3285			0.4 ± 0.3		0.9 ± 0.8	0.9 ± 0.8
β-MCA	3306	14.6 ± 0.8	12.7 ± 6.7	33.1 ± 9.1	25.3 ± 16.8	60.1 ± 0.4	17.1 ± 3.4
Δ22-β-MCA	3333	0.5 ± 0.1	0.1 ± 0.1	3.2 ± 1.4	1.5 ± 1.1	7.2 ± 3.4	
ω-MCA	3405	12.6 ± 3.4	0.4 ± 0.1	6.9 ± 2.8	1.6 ± 1.6	2.3 ± 2.3	1.4 ± 1.4
Ol-/diol-ones		1.9 ± 1.4	0.4 ± 0.1	6.1 ± 0.7	3.9 ± 1.7	3.9 ± 1.0	2.1 ± 2.1
Polyhydroxylated							
1β,3α,12α-Triol	3285	0.8 ± 0.9	7.4 ± 6.1	0.5 ± 0.2	4.5 ± 1.9		
3α,6β,7α,12α-Tetrol	3216						0.7 ± 0.2
3α,6β,7β,12α-Tetrol	3253						0.5 ± 0.5
3α,6α,7α,12α-Tetrol	3287						2.2 ± 2.2
1β,3α,7α,12α-Tetrol	3299		1.7 ± 1.9	0.9 ± 0.6	1.5 ± 1.4	0.8 ± 0.4	0.2 ± 0.2
3α,7α,12α,22-Tetrol	3319		2.6 ± 1.7	1.6 ± 0.8	2.7 ± 3.0	0.6 ± 0.1	6.0 ± 5.2
3α,7α,12α,23-Tetrol	3346					0.7 ± 0.3	2.2 ± 2.3
1β,3α,6β,7β-Tetrol	3376						
3α,6β,7β,22-Tetrol	3387					4.0 ± 2.7	5.4 ± 0.3
2β,3α,7α,12α-Tetrol	3431			0.5 ± 0.1	2.8 ± 1.9	0.8 ± 0.5	1.2 ± 1.7
3α,4β,7α,12α-Tetrol	3444			0.4 ± 0.3	0.3 ± 0.3		7.7 ± 3.3
3,6,7,12,22-Pentol	3500					3.1 ± 1.1	6.7 ± 4.1
Other polyols						0.9 ± 0.8	1.4 ± 1.4
Total polyols			4.3	3.4	7.3	10.9	29.9

Values are expressed as means ± SD (n = 5 mice per group). CA, cholic acid; CBDL, common bile duct ligation; CDCA, chenodeoxycholic acid; DCA, deoxycholic acid; $Fxr^{-/-}$, farnesoid X receptor knockout; HCA, hyocholic acid; LCA, lithocholic acid; MCA, muricholic acid; RI, retention index; UDCA, ursodeoxycholic acid.

[a] $P < 0.05$, naive versus CBDL.

[b] $P < 0.05$, wild type versus $Fxr^{-/-}$.

TABLE 2. Serum and biliary bile acids in wild-type and *Fxr*$^{-/-}$ mice during biliary obstruction

		Serum				Bile			
		Naive		7 d CDBL		Naive		7 d CDBL	
Bile Acids	RI	Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$
		% of total							
Total (μmol/ml)		0.009 ± 0.002	0.014 ± 0.003	0.84 ± 0.50[a]	0.21 ± 0.18[a]	57.1 ± 12.9	72.7 ± 5.2[b]	67.4 ± 15.6	106.4 ± 28.6
Primary and secondary									
LCA	3107								
allo-CA	3173			1.8 ± 0.3	0.3 ± 0.4				
DCA	3177	0.5 ± 0.1				1.5 ± 1.8	0.2 ± 0.2	1.0 ± 1.0	
CDCA	3195			4.5 ± 0.4	0.7 ± 0.5			0.6 ± 0.1	
α-MCA	3200	14.0 ± 0.1		6.3 ± 0.1	2.9 ± 2.6	3.4 ± 0.6	0.4 ± 0.2	2.9 ± 0.4	0.2 ± 0.2
CA	3214	13.9 ± 12.6	53.0 ± 6.9	26.1 ± 11.0	46.1 ± 2.5	61.7 ± 0.1	77.3 ± 4.9	28.7 ± 1.4	83.1 ± 0.4
UDCA	3230	0.7 ± 0.8	0.3 ± 0.4	0.6 ± 0.3	0.3 ± 0.4	1.0 ± 0.1	0.1 ± 0.1	0.3 ± 0.3	0.2 ± 0.3
HCA	3285					0.5 ± 0.2	0.3 ± 0.4		
β-MCA	3306	22.0 ± 5.5	12.2 ± 2.8	24.2 ± 2.3	2.2 ± 1.2	14.8 ± 0.5	5.8 ± 3.3	42.1 ± 1.7	6.9 ± 1.9
Δ22-β-MCA	3333			2.4 ± 1.0					
ω-MCA	3405	13.7 ± 5.0	7.1 ± 1.5	3.1 ± 1.6		5.5 ± 0.5	2.4 ± 1.6	1.4 ± 0.2	
Ol-/diol-ones		47.8 ± 8.4	14.1 ± 12.0	23.9 ± 3.0	4.1 ± 1.7	5.0 ± 1.4	7.8 ± 3.1	20.4 ± 5.8	5.0 ± 0.6
Polyhydroxylated									
1β,3α,12α-Triol	3285								
3α,6β,7α,12α-Tetrol	3216								
3α,6β,7β,12α-Tetrol	3253								
3α,6α,7α,12α-Tetrol	3287				0.5 ± 0.1				
1β,3α,7α,12α-Tetrol	3299			2.4 ± 1.7	9.4 ± 1.5				
3α,7α,12α,22-Tetrol	3319				7.5 ± 7.1		1.0 ± 0.2		0.5 ± 0.2
3α,7α,12α,23-Tetrol	3346				5.8 ± 1.8				
1β,3α,6β,7β-Tetrol	3376								
3α,6β,7β,22-Tetrol	3387			2.0 ± 1.7			1.8 ± 1.3		1.1 ± 0.7
2β,3α,7α,12α-Tetrol	3431		0.7 ± 0.8		3.0 ± 1.6				
3α,4β,7α,12α-Tetrol	3444		12.6 ± 11.4	2.2 ± 0.1	14.8 ± 3.2		2.9 ± 3.8	1.1 ± 0.3	3.0 ± 1.8
3,6,7,12,22-Pentol	3500				2.6 ± 1.2				
Other polyols									
Total polyols			13.3	6.6	43.6		5.7	1.1	4.6

Values are expressed as means ± SD (serum, n = 5 mice per group; bile, n = 3 mice per group).
[a] $P < 0.05$, naive versus CBDL.
[b] $P < 0.05$, wild type versus *Fxr*$^{-/-}$.

TABLE 3. Urine bile acids in wild-type and *Fxr*$^{-/-}$ mice during biliary obstruction

Bile Acids	RI	Naive		3 d CBDL		5 d CDBL		7 d CBDL	
		Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$	Wild Type	*Fxr*$^{-/-}$
		% of total							
Total (μmol/24 h)		0.008 ± 0.003	0.033 ± 0.027	0.33 ± 0.03[a]	3.16 ± 0.91[a,b]	1.23 ± 0.71[a]	4.05 ± 0.40[a,b]	0.90 ± 0.76	0.29 ± 0.19
Primary and secondary									
LCA	3107								
allo-CA	3173			3.7 ± 2.2	0.4 ± 0.2	6.7 ± 3.8	0.8 ± 0.9	0.8 ± 0.8	0.2 ± 0.2
DCA	3177								
CDCA	3195								
α-MCA	3200			0.4 ± 0.3	1.0 ± 1.3	3.8 ± 2.7	1.1 ± 1.1	0.6 ± 0.5	0.2 ± 0.2
CA	3214	+	+	39.1 ± 1.8	34.8 ± 16.9	10.5 ± 0.4	13.9 ± 1.0	6.5 ± 3.4	0.2 ± 0.2
UDCA	3230								
HCA	3285								
β-MCA	3306	+		13.3 ± 3.8		21.4 ± 1.0		3.1 ± 2.7	
Δ22-β-MCA	3333								
ω-MCA	3405								
Ol-/diol-ones		+	+						
Polyhydroxylated									
1β,3α,12α-Triol	3285								
3α,6β,7α,12α-Tetrol	3216						3.2 ± 1.6	9.2 ± 3.1	7.4 ± 5.2
3α,6β,7β,12α-Tetrol	3253								
3α,6α,7α,12α-Tetrol	3287		+	2.7 ± 1.3	3.6 ± 0.5	2.2 ± 1.7	7.8 ± 0.8	3.8 ± 3.1	4.9 ± 2.1
1β,3α,7α,12α-Tetrol	3299		+	4.3 ± 2.1	21.7 ± 6.2	6.2 ± 1.2	18.3 ± 3.1	8.3 ± 5.1	21.4 ± 5.1
3α,7α,12α,22-Tetrol	3319		+	2.8 ± 2.0	6.4 ± 4.4	1.9 ± 0.2	3.8 ± 1.4	2.8 ± 1.9	11.7 ± 4.3
3α,7α,12α,23-Tetrol	3346			2.3 ± 1.3	7.6 ± 3.0		15.2 ± 0.2	4.5 ± 4.1	21.7 ± 7.8
1β,3α,6β,7β-Tetrol	3376		+	10.2 ± 2.6	9.7 ± 4.4	11.2 ± 5.7	3.9 ± 0.5	11.0 ± 5.6	4.0 ± 3.1
3α,6β,7β,22-Tetrol	3387			6.8 ± 0.7	4.2 ± 1.9	10.9 ± 3.4	4.7 ± 3.8	19.1 ± 5.8	9.0 ± 5.2
2β,3α,7α,12α-Tetrol	3431				2.3 ± 2.1				0.3 ± 0.3
3α,4β,7α,12α-Tetrol	3444			0.9 ± 0.3	1.0 ± 0.4	5.8 ± 2.0	10.5 ± 1.0	11.1 ± 1.1	1.6 ± 1.2
3,6,7,12,22-Pentol	3500			3.4 ± 0.2	5.9 ± 2.1	6.8 ± 1.9	6.4 ± 3.1	10.0 ± 5.7	6.8 ± 4.9
Other polyols				10.1 ± 3.2	4.0 ± 0.7	12.5 ± 3.8	10.4 ± 6.6	9.2 ± 5.7	10.6 ± 2.3
Total polyols				43.5	66.6	57,6	84.2	89.0	99.4

Values are expressed as means ± SD (n = 5 mice per group).
[a] $P < 0.05$, naive versus CBDL.
[b] $P < 0.05$, wild type versus *Fxr*$^{-/-}$.

hydrocarbons with 30 (RI = 3000) and 36 (RI = 3600) carbon atoms.

Preparation of total RNA and determination of mRNA levels by real-time PCR

The preparation of total RNA, reverse transcription into cDNA, TaqMan® real-time PCR, and primer/probe sequences for *Cyp2b10* and *Cyp3a11* were described previously (13, 14).

Preparation of kidney membranes and analysis of renal bile acid transporter protein levels by Western blotting

Kidney membranes were prepared and Mrp2–Mrp4 and organic anion-transporting polypeptide 1 (Oatp1) protein levels were determined as described previously (15). Apical sodium-dependent bile acid transporter (Asbt) protein expression levels were determined using a polyclonal rabbit antibody against Asbt (dilution, 1:2,500; kindly provided by Dr. Paul A. Dawson, Wake Forest University School of Medicine, Winston-Salem, NC). Blots were reprobed with an anti-β-actin antibody (1:5,000; Sigma) to confirm the specificity of changes in transporter protein levels. Apical membrane targeting of Asbt was confirmed by immunohistochemistry (13) using the Asbt antibody provided by Dr. Dawson. No differences were found between wild-type and $Fxr^{-/-}$ mice, either in naive or 7 d bile duct-ligated animals (data not shown).

Statistical analysis

In each group, three to five animals were studied. Data are reported as arithmetic means ± SD. For statistical analysis, ANOVA with Bonferroni posttest testing (for multiple comparisons) or Student's *t*-test (for single time points of two groups) was used with the SigmaStat statistics program (Jandel Scientific, San Rafael, CA). $P < 0.05$ was considered significant.

RESULTS

Quantitative changes in bile acid levels during obstructive cholestasis

Liver tissue bile acid levels increased during CBDL; in wild-type mice, from 0.07 ± 0.04 μmol/g at baseline to 1.00 ± 0.06 μmol/g at day 3 and to 0.92 ± 0.43 μmol/g at day 7 after CBDL. Table 1 shows that liver tissue bile acid levels in $Fxr^{-/-}$ mice did not differ from those in wild-type mice at baseline (0.10 ± 0.07 μmol/g) but were significantly lower at day 7 after CBDL (0.36 ± 0.016 μmol/g; $P < 0.05$), as described previously (7).

Serum bile acid levels did not differ between genotypes at baseline (0.009 ± 0.002 μmol/ml in wild-type mice and 0.014 ± 0.003 μmol/ml in $Fxr^{-/-}$ mice). However, at day 7 after CBDL, serum bile acid levels were ~4-fold higher in wild-type mice (0.84 ± 0.50 μmol/ml) compared with $Fxr^{-/-}$ mice (0.21 ± 0.18 μmol/ml; $P < 0.05$).

Biliary bile acid levels at baseline were higher in $Fxr^{-/-}$ mice (72.7 ± 5.2 μmol/ml vs. 57.1 ± 12.9 μmol/ml in wild-type mice; $P < 0.05$), as described by Kok et al. (6). Also after CBDL, biliary bile acid levels were higher in $Fxr^{-/-}$ mice (106.4 ± 28.6 μmol/ml vs. 67.4 ± 15.6 μmol/ml in wild-type mice) (**Table 2**), but this difference did not reach statistical significance.

Urinary bile acid excretion rates increased from ~0.01 to 0.03 μmol/24 h in both naive genotypes to 0.33 ± 0.3 μmol/24 h in wild-type mice and to 3.16 ± 0.91 μmol/24 h in $Fxr^{-/-}$ mice at day 3 after CBDL ($P < 0.05$) and increased further to 1.23 ± 0.71 and 4.05 ± 0.40 μmol/24 h, respectively, at day 5 after CBDL (**Table 3**). The excretion rates at days 3 and 5, respectively, were significantly different ($P < 0.05$) between genotypes. At day 7 after CBDL, total bile acid excretion declined, to 0.90 ± 0.76 μmol/24 h in wild-type mice and to 0.29 ± 0.19 μmol/24 h in $Fxr^{-/-}$ mice.

The higher urinary bile acid elimination in $Fxr^{-/-}$ mice could principally be explained by *i*) excretion of compounds that might differ from those in wild-type mice, *ii*) differences in expression levels of bile acid-transporting membrane proteins, *iii*) an increased bile acid production rate, or a combination of these factors. To answer these questions, we performed *i*) a comprehensive bile analysis and *ii*) a comparison of putative renal bile acid transport proteins. The total urinary excretion of bile acids in CBDL was assumed to reflect total bile acid synthesis, which would be expected to be increased in the absence of FXR.

ES-MS is a highly sensitive method for the detection of different types of conjugated bile acids. Screening by this method did not reveal the presence of bile acid sulfates or glucuronides in liver, serum, bile, or urine. The analyses were performed both before and after cholylglycine hydrolysis to exclude the suppression of low-abundance anions by the predominant ions of taurine conjugates.

ES-MS revealed differences in the proportion of polyhydroxylated bile acids between FXR genotypes. In naive wild-type liver, only anions at *m/z* 498 and 514, indicative of taurine-conjugated dihydroxylated and trihydroxylated bile acids, at a ratio of 1:4, were found. Spectra of liver from naive $Fxr^{-/-}$ mice showed an anion at *m/z* 530 of minor intensity (<10%), indicative of taurine-conjugated tetrahydroxylated bile acids. This particular ion gained in relative intensity in the spectra of liver and serum after CBDL and became most abundant in urine. The relative intensity of *m/z* 530, compared with *m/z* 514, was always higher in $Fxr^{-/-}$ mice than in wild-type mice. There was an additional ion at *m/z* 546, indicative of taurine-conjugated pentahydroxylated bile acids, in spectra of liver (**Fig. 1**) and serum from $Fxr^{-/-}$ mice at day 7 of CBDL. This ion was also found to various extents in spectra of urine from both genotypes. Together, the ES-MS analyses excluded significant phase II (sulfation and glucuronidation) detoxification of bile acids in mice with obstructive cholestasis but indicated that phase I detoxification (hydroxylation) was enhanced in $Fxr^{-/-}$ mice. Ions indicative of hydroxylated C-27 bile acid precursors (8) were not observed in any material studied.

Qualitative changes in bile acid levels during obstructive cholestasis

GC-MS analysis data of the bile acid composition of wild-type and $Fxr^{-/-}$ mice are shown for liver in Table 1, for serum and bile in Table 2, and for urine in Table 3. The

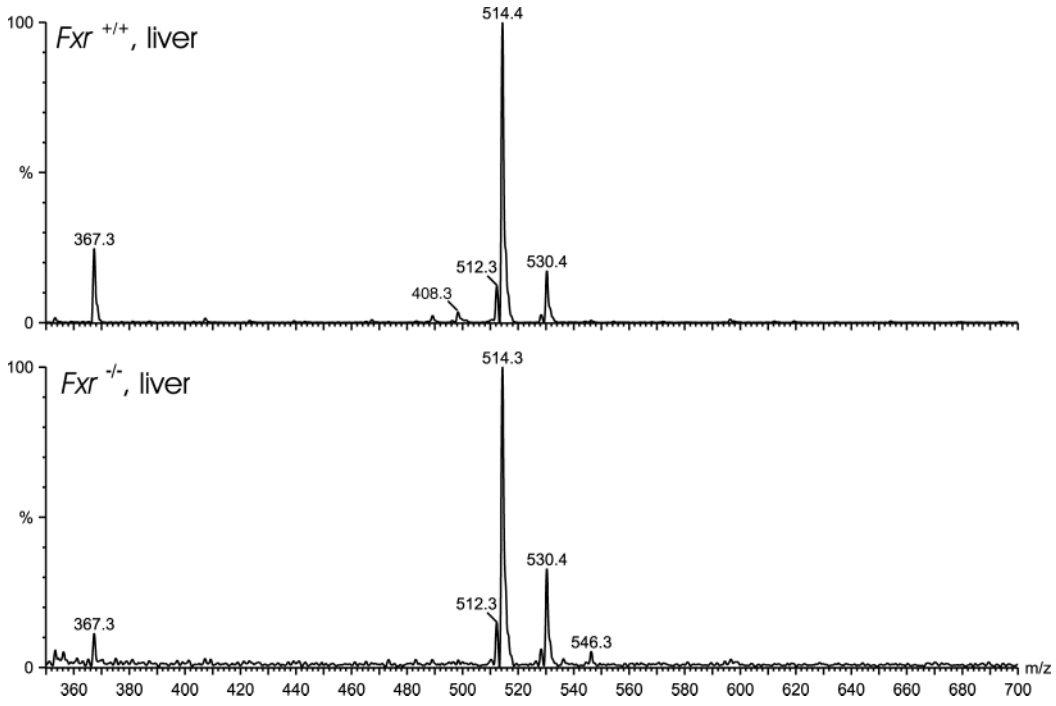

Fig. 1. Formation of tetrahydroxylated and pentahydroxylated bile acids in biliary obstruction. Electrospray mass spectra of extracts of crude liver homogenates of wild-type mice (upper spectrum) and farnesoid X receptor knockout ($Fxr^{-/-}$) mice (lower spectrum) after 7 days of common bile duct ligation (CBDL). *m/z* 498, 514, 530, and 546 represent deprotonated molecules of taurine-conjugated dihydroxylated, trihydroxylated, tetrahydroxylated, and pentahydroxylated bile acids, respectively.

relative amount of the major primary bile acid CA decreased significantly in the liver of wild-type mice, from 39% to 12% at day 7 of CBDL, whereas the contribution of CA remained unchanged in $Fxr^{-/-}$ mice, as we described previously (7). The relative amount of β-MCA increased continuously in livers of wild-type mice but decreased in livers of $Fxr^{-/-}$ mice (Table 1). The decline of β-MCA was even more pronounced in serum of cholestatic $Fxr^{-/-}$ mice (Table 2).

As expected, the secondary bile acids lithocholic acid (LCA) and deoxycholic acid (DCA) virtually disappeared during obstructive cholestasis (Tables 1–3). This was paralleled by the disappearance of 1β-hydroxy-DCA, in particular from livers of $Fxr^{-/-}$ mice. There was also some Δ^{22}-β-MCA in livers and serum of naive and cholestatic wild-type mice, indicating partial β-oxidation as a pathway for bile acid metabolism not only in rats (9) but also in mice. Notably, Δ^{22}-β-MCA was not found in livers and serum of $Fxr^{-/-}$ mice and was not excreted in bile and urine of any of the genotypes (Tables 1–3). The levels of major murine bile acids in bile of naive animals were, within statistical margins, the same as those found by Kok et al. (6) (Table 2). The larger relative amount of biliary CA in $Fxr^{-/-}$ mice increased even more after CBDL. This might be attributable to the lack of FXR-mediated inhibition of cholesterol 7α-hydroxylase in the classical pathway leading to CA. Another new finding was the presence of small amounts of tetrahydroxylated bile acids in bile of naive $Fxr^{-/-}$ mice and in bile of both genotypes after 7 days of CBDL (Table 2).

Polyhydroxylated bile acids

The most important differences in bile acid profiles between wild-type and $Fxr^{-/-}$ mice were observed particularly in liver, serum, and urine as higher abundances of polyhydroxylated compounds in $Fxr^{-/-}$ mice. The formation of these compounds was obviously activated already in naive $Fxr^{-/-}$ mice, as seen by the occurrence of 4.3, 5.7, and 12.2% of tetrols in liver, bile, and serum, respectively, of these animals (Tables 1, 2). Tetrols were also found in the urine of naive $Fxr^{-/-}$ mice, but the total urinary excretion at baseline was too low for a quantitative analysis. During cholestasis, the relative amounts of polyhydroxylated bile acids were always higher in $Fxr^{-/-}$ mice compared with their wild-type littermates, and after 7 days of CBDL, these acids constituted ~30, 45, and 100% of the total bile acids in liver, serum, and urine, respectively, from $Fxr^{-/-}$ mice (Tables 1–3).

The major part of polyhydroxylated bile acids consisted of hydroxylation products of CA, carrying an additional hydroxyl group in the 1β, 2β, 6α, 6β, 22, or 23 position. Mass spectra of the latter two compounds are shown in **Fig. 2**. There was also a pentahydroxylated compound found in liver, serum, and urine that was tentatively identified as CA hydroxylated in both the 6 and 22 positions.

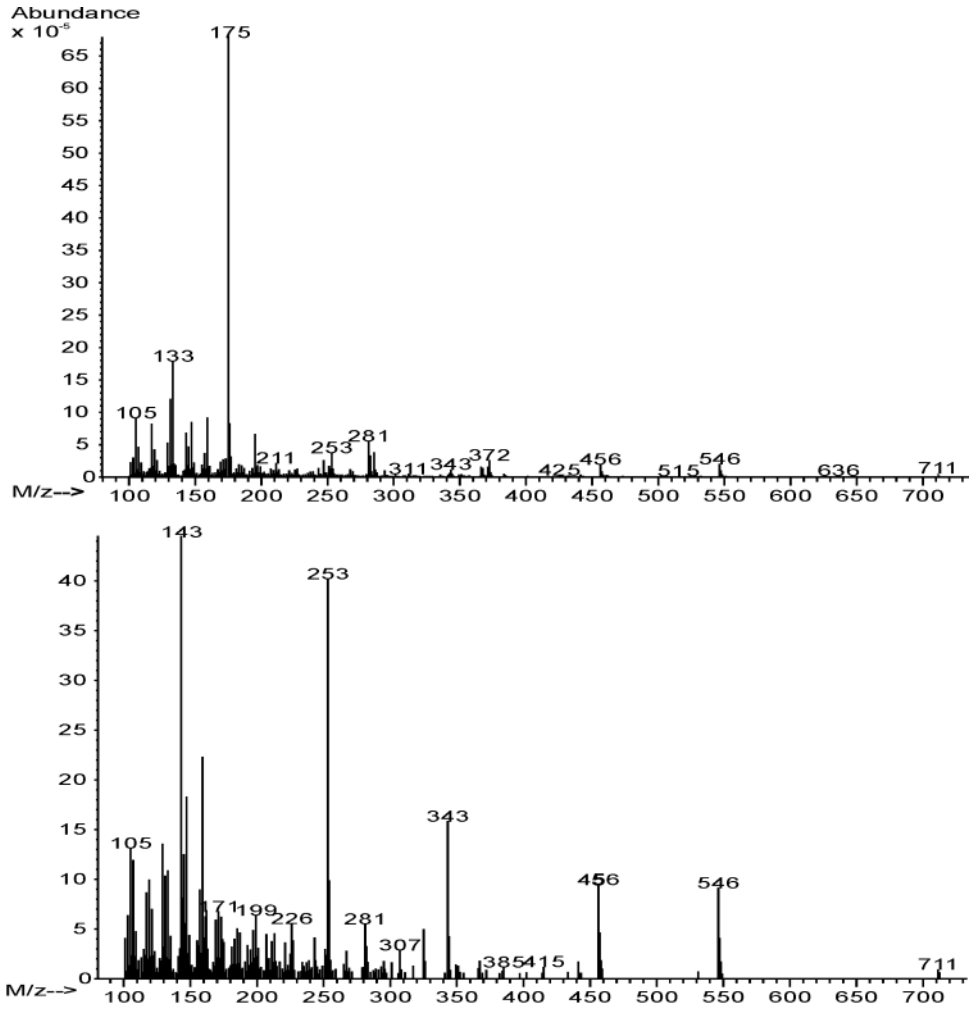

Fig. 2. Formation of 22- and 23-hydroxylated products of cholic acid (CA) in biliary obstruction. Electron impact mass spectra of methyl ester trimethylsilyl ether derivatives of 3α,7α,12α,22-5β-cholan-24-oic acid (upper spectrum) and 3α,7α,12α,23-5β-cholan-24-oic acid (lower spectrum). The ions at *m/z* 711 are formed by the loss of a methyl group from the molecular ions.

Thus, after 7 days of CBDL, 60–100% of the total polyhydroxylated bile acids found in liver, serum, and urine of $Fxr^{-/-}$ mice were hydroxylation products of CA, possibly reflecting the lack of FXR-mediated inhibition of the biosynthetic pathway to CA.

mRNA levels of *Cyp2b10* and *Cyp3a11*

To determine whether the increase in bile acid hydroxylation is also accompanied by an increase in cytochromes P450, mRNA levels of *Cyp2b10* and *Cyp3a11* in the liver and kidney were determined. Two-fold higher basal expression levels of *Cyp2b10* ($P < 0.05$) and *Cyp3a11* (not significant) were found in livers of naive $Fxr^{-/-}$ mice (**Fig. 3**). After 3 d of CBDL, *Cyp2b10* gene expression levels were increased 10-fold in both genotypes ($P < 0.05$ compared with naive animals; not significant between genotypes). At 7 d of CBDL, *Cyp2b10* gene expression levels had returned to the baseline in both wild-type and $Fxr^{-/-}$ mice (Fig. 3). In contrast, *Cyp3a11* expression levels in $Fxr^{-/-}$ mice were significantly higher at both 3 and 7 d of CBDL compared with those in wild-type mice (3.5- and 4.0-fold, respectively) (Fig. 3). No significant *Cyp2b10* or *Cyp3a11* gene expression was found in the kidney of naive or cholestatic animals. These data indicate the involvement of hepatic *Cyp3a11* in the hydroxylation of bile acids.

Renal bile acid transporter expression profiles

To investigate whether changes in renal bile acid export (Mrp2 and Mrp4) and reuptake transporters (Oatp1 and Asbt) could account for the differences in urinary bile acid clearance, protein levels of the respective transporters were determined. In contrast to the situation in rats (15), no changes were observed in the expression levels of renal Mrp2 and Asbt (**Fig. 4**). Also, Oatp1 remained unchanged. However, renal Mrp4 was significantly induced in both genotypes after 3 days of CBDL, 2.9-fold in wild-type mice and 2.3-fold in $Fxr^{-/-}$ mice. Mrp3 was not expressed or induced in the kidney of any of the genotypes (data not shown). Notably, significant differences in baseline levels were not observed in any of the renal transporters studied

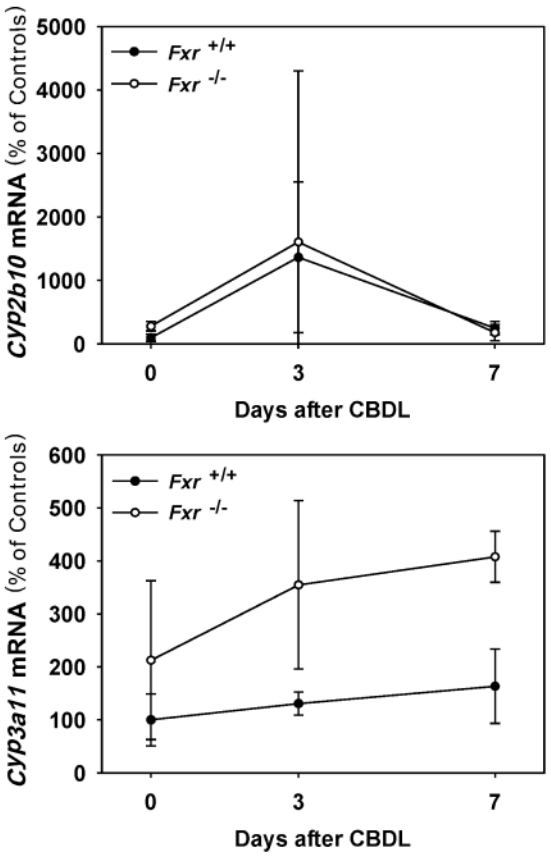

Fig. 3. Hepatic mRNA levels of *Cyp2b10* (upper graph) and *Cyp3a11* (lower graph) in biliary obstruction. Increases of *Cyp2b10* mRNA expression levels are observed in both genotypes at day 3. In contrast, mRNA expression levels of *Cyp3a11* differ significantly between wild-type and $Fxr^{-/-}$ mice in obstructive cholestasis on both days 3 and 7, which is consistent with the larger proportion of polyhydroxylated bile acids formed in $Fxr^{-/-}$ mice. Controls were naive $Fxr^{-/-}$ wild-type mice.

(data not shown). Together, these data suggest a coordinated alternative excretory pathway for bile acids via hepatic (7) and renal Mrp4.

DISCUSSION

This study presents detailed analyses of bile acids in liver, serum, bile, and urine of cholestatic $Fxr^{-/-}$ mice and parallel measurements of the expression of major renal bile acid transporters. Our data provide an explanation for the surprising finding that $Fxr^{-/-}$ mice eliminate the systemic and hepatic bile acid load more rapidly in biliary obstruction than their wild-type littermates, despite their inability to upregulate the canalicular bile acid export pump, Bsep. Instead, this inability may result in the efflux of bile acids from the hepatocytes into the blood for subsequent excretion into urine.

$Fxr^{-/-}$ mice most efficiently synthesize polyhydroxylated bile acids, and the majority of these compounds are hydroxylation products of CA, the most abundant bile acid in mice. This prototype phase I detoxification reaction is of particular importance because $Fxr^{-/-}$ mice, in contrast to their littermates, are unable to downregulate *Cyp8b1* (6, 7) and actually were shown to have a 2-fold increased synthesis rate of CA (6). Thus, the metabolism we describe here preferably detoxifies CA, which is the major bile acid retained in cholestasis and has a notorious toxicity in feeding experiments on mice (4, 5).

This mechanism is of obvious importance in $Fxr^{-/-}$ mice. We estimate that $Fxr^{-/-}$ mice, with a mean urinary bile acid excretion rate of ~2 μmol/day during the first 7 days after CBDL, eliminate ~50% of the bile acid load during obstructive cholestasis into urine. This estimation is based on recent data on the total bile acid pool size and CA production rates in $Fxr^{-/-}$ mice with normal intestinal bile acid absorption (6).

Although sulfation and glucuronidation are of relevance in the urinary elimination of bile acids in humans, our ES-MS data show that significant amounts of sulfates or glucuronides are not present in CBDL mice. This is in agreement with data obtained in rats using a similar experimental approach (16). Thus, these phase II detoxification mechanisms are most likely of no importance in rodents with obstructive cholestasis. The induction of hydroxysteroid sulfotransferase in LCA-fed $Fxr^{-/-}$ mice described recently (17) may be specific for LCA but apparently does not lead to an increased sulfation of other bile acids. LCA is a very minor compound in naive $Fxr^{-/-}$ mice and is virtually absent in obstructive cholestasis, as shown in this study and a previous study (6).

Our gene expression studies support the involvement of Cyp3a11 in the hydroxylation of CA. The expression of pregnane X receptor (PXR)-dependent Cyp3a11 (18, 19) was significantly more increased after 3 and 7 d of CBDL in $Fxr^{-/-}$ mice than in wild-type mice. In agreement with previous reports (7, 20), we found enhanced expression of *Cyp3a11* already in naive animals, but this difference was not statistically different. Cyp3a11 is homologous to human CYP3A4, which has been shown in both in vitro (18, 21) and in vivo (21) experiments to hydroxylate bile acids in the 1β, 6α, and 6β positions. *Cyp2b10* gene expression was also studied, because its human homolog, CYP2B6, can hydroxylate 5β-cholestane-3α,7α,12α-triol, a minor CA precursor, in the 25 position, although to a much lesser extent than CYP3A4 (22). We did not find significant differences in *Cyp2b10* gene expression levels in cholestatic mice of either genotype, supporting the assumption that this enzyme is not involved in the bile acid hydroxylations seen in this study. Increased formation of polyhydroxylated bile acids was also found in naive wild-type Swiss albino mice given constitutive androstane receptor (CAR)- and PXR-stimulating agents (14). However, the structures of these bile acids were not established.

The formation of hydroxylation products is a prototype phase I detoxification reaction. A larger number of hydroxyl groups are associated with higher hydrophilicity and usually with lower toxicity. In the case of bile acid metabolism in humans, this form of detoxification involves CYP3A4 in a feed-forward manner. Although LCA has been shown to bind to and activate the nuclear

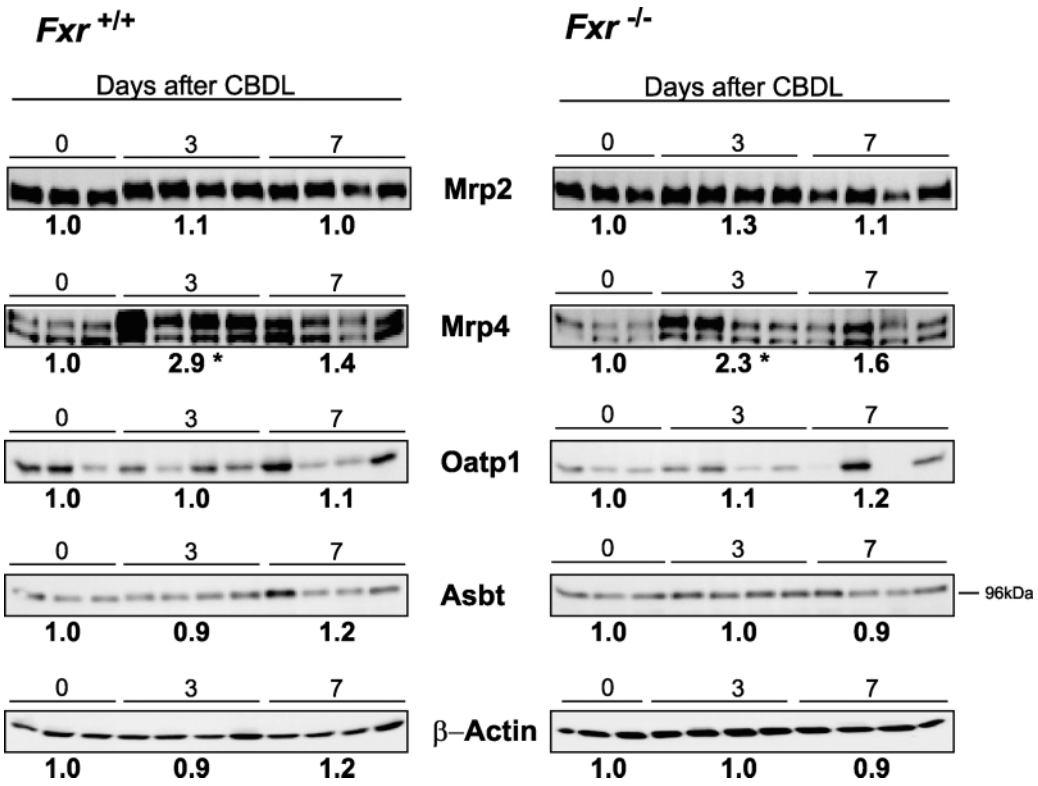

Fig. 4. Effects of biliary obstruction on putative renal bile salt transporters in wild-type and *Fxr*$^{-/-}$ mice. Kidney membranes were isolated from wild-type and *Fxr*$^{-/-}$ mice before (day 0) and after 3 and 7 days of CDBL and analyzed by Western blotting as described in Experimental Procedures. On day 3 of CBDL, a significant induction of multidrug resistance-related protein 4 (Mrp4) was observed in both genotypes ($P < 0.05$). Asbt, apical sodium-dependent bile acid transporter; Oatp1, organic anion-transporting polypeptide 1.

receptor PXR that enhances CYP3A4 expression, resulting in the formation particularly of 6α-hydroxylated bile acids (18), this mechanism has also been shown for CBDL mice with undetectable LCA levels. This suggests that accumulation of bile acids other than LCA also could activate this distinct detoxification pathway (23).

Because polyhydroxylated bile acids did not appear in great amounts in bile but were found almost exclusively in serum and urine, an efficient export of these compounds via hepatic basolateral and renal tubular export seems most likely. The rapid declines in liver tissue and systemic bile acid levels were paralleled with an enhanced expression of Mrp4 in both the liver (5) and the kidney. Thus, it is attractive to speculate that these two adaptive mechanisms work together (i.e., that Mrp4 actively excretes tetrahydroxylated bile acids both from the liver at the basolateral side and from the kidney at the apical side). However, we did not find the same relationships between enhanced bile acid hydroxylation and enhanced Mrp4 expression in wild-type and *Fxr*$^{-/-}$ genotypes, which indicates that the changes of Mrp4 expression and bile acid hydroxylation are differently regulated in the two genotypes. Whether passive glomerular filtration or another, as yet undefined transport system contributes to the efficient renal clearance of polyhydroxylated bile acid metabolites remains to be investigated. The fact that excretion of these compounds into bile did not increase during CBDL argues against Mrp2 as a potential transporter. Mrp3, which is upregulated to a greater extent in the liver of CBDL *Fxr*$^{-/-}$ mice (5), is not even expressed/induced in the kidney. Preliminary results of renal Mrp6 expression after bile acid treatment indicate that this tubular apical transport system also did not contribute to the increased urinary bile acid excretion (8). Furthermore, decreases in renal Oatp1 and Asbt, which would be predicted to enhance renal bile acid output by reduced bile acid reuptake, were not detected in CBDL mice, in contrast to the downregulation of renal Asbt in CBDL rats (16).

The mechanisms by which *Fxr*$^{-/-}$ mice protect themselves against bile acid toxicity more efficiently than their wild-type littermates may have implications for our understanding of protective mechanisms in human cholestasis. The elimination of bile acids in urine may be considerable in patients with extrahepatic or intrahepatic cholestasis (24). Most of the hydroxylation products of CA that we have found to increase substantially in *Fxr*$^{-/-}$ mice have been described in urine of humans with various cholestatic liver diseases (12, 25–27). In fact, it was concluded that the occurrence of these compounds most typically distin-

guished normal from cholestatic conditions (23), and it was suggested that cholestatic infants with a good capacity for hydroxylation reactions have a better prognosis (24). Notably, in these studies of humans, the tetrahydroxylated bile acids were amidated but otherwise unconjugated, as found here in the mouse. Large amounts of sulfated bile acids are excreted in human urine during cholestasis, but sulfation is most active with less polar monohydroxy and dihydroxy bile acids. Besides chenodeoxycholic acid, these include the secondary bile acids LCA and DCA (24), which also decrease in humans with time of cholestasis as a result of decreased reabsorption from the intestine and enhanced 6α-hydroxylation (12, 25–28). Glucuronidation then emerges as a specific detoxification of 6α-hydroxylated bile acids (29).

Our data show that *Fxr*$^{-/-}$ mice have higher biliary bile acid concentrations than their wild-type littermates not only at baseline, as also shown by Kok et al. (6), but also after CBDL. However, this does not necessarily indicate larger amounts of bile acids in the bile duct. The latter is the case only in wild-type mice, in which we found a significant increase in bile duct pressure in CBDL as a result of continuous bile acid pumping via upregulated Bsep (7). In *Fxr*$^{-/-}$ mice, biliary bile acids may be excreted via other as yet undefined bile acid transporters. This is probably also the case in *Bsep* (*spgp*) knockout mice (30, 31).

In summary, we describe hydroxylation reactions to be the first line of defense against bile acid toxicity in biliary obstruction. We speculate that the more rapid and efficient hydroxylation in *Fxr*$^{-/-}$ mice, together with enhanced Mrp4 expression, favor these animals for studies of treatments aimed at an enhancement of cytochrome P450-dependent phase I detoxification reactions [e.g., by the administration of PXR ligands such as pregnenolone-16α-carbonitrile (PCN) or statins].

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REFERENCES

1. Russell, D. W. 2003. The enzymes, regulation, and genetics of bile acid synthesis. *Annu. Rev. Biochem.* **72:** 137–174.
2. Trauner, M., and J. L. Boyer. 2003. Bile salt transporters: molecular characterization, function, and regulation. *Physiol. Rev.* **83:** 633–671.
3. Chiang, J. Y. 2004. Regulation of bile acid synthesis: pathways, nuclear receptors, and mechanisms. *J. Hepatol.* **40:** 539–551.
4. Sinal, C. J., M. Tohkin, M. Miyata, J. M. Ward, G. Lambert, and F. J. Gonzalez. 2000. Targeted disruption of the nuclear receptor FXR/BAR impairs bile acid and lipid homeostasis. *Cell.* **102:** 731–744.
5. Zollner, G., P. Fickert, A. Fuchsbichler, D. Silbert, M. Wagner, S. Arbeiter, F. J. Gonzalez, H. U. Marschall, K. Zatloukal, H. Denk, et al. 2003. Role of nuclear bile acid receptor, FXR, in adaptive ABC transporter regulation by cholic and ursodeoxycholic acid in mouse liver, kidney and intestine. *J. Hepatol.* **39:** 480–488.
6. Kok, T., C. V. Hulzebos, H. Wolters, R. Havinga, L. B. Agellon, F. Stellaard, B. Shan, M. Schwarz, and F. Kuipers. 2003. 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. *J. Biol. Chem.* **278:** 41930–41937.
7. Wagner, M., P. Fickert, G. Zollner, A. Fuchsbichler, D. Silbert, O. Tsybrovskyy, K. Zatloukal, G. L. Guo, J. D. Schuetz, F. J. Gonzalez, et al. 2003. Role of farnesoid X receptor in determining hepatic ABC transporter expression and liver injury in bile duct-ligated mice. *Gastroenterology.* **125:** 825–838.
8. Zollner, G., E. Halilbasic, M. Wagner, P. Fickert, D. Silbert, J. Gumhold, A. Fuchsbichler, K. Zatlaukal, H. U. Marschall, H. Denk, et al. 2004. Cholic acid (CA) and ursodeoxycholic acid (UDCA) exert different effects on hepatic and renal expression of Mrp4 and Mrp6, two novel ABC transporters. *Gastroenterology.* **126 (Suppl. 2):** A-16.
9. Setchell, K. D., H. Yamashita, C. M. Rodrigues, N. C. O'Connell, B. T. Kren, and C. J. Steer. 1995. Delta 22-ursodeoxycholic acid, a unique metabolite of administered ursodeoxycholic acid in rats, indicating partial beta-oxidation as a major pathway for bile acid metabolism. *Biochemistry.* **34:** 4169–4178.
10. Marschall, H. U., U. Broome, C. Einarsson, G. Alvelius, H. G. Thomas, and S. Matern. 2001. Isoursodeoxycholic acid: metabolism and therapeutic effects in primary biliary cirrhosis. *J. Lipid Res.* **42:** 735–742.
11. Lawson, A. M., and K. D. Setchell. 1988. Mass spectrometry of bile acids. *In* The Bile Acids. Vol. 4. Chemistry, Physiology, and Metabolism. K. D. Setchell, D. Kritchevsky, and P. P. Nair, editors. Plenum Press, New York. 167–267.
12. Bremmelgaard, A., and J. Sjovall. 1980. Hydroxylation of cholic, chenodeoxycholic, and deoxycholic acids in patients with intrahepatic cholestasis. *J. Lipid Res.* **21:** 1072–1081.
13. Fickert, P., A. Fuchsbichler, M. Wagner, G. Zollner, A. Kaser, H. Tilg, R. Krause, F. Lammert, C. Langner, K. Zatloukal, et al. 2004. Regurgitation of bile acids from leaky bile ducts causes sclerosing cholangitis in Mdr2 (Abcb4) knockout mice. *Gastroenterology.* **127:** 261–274.
14. Wagner, M., E. Halilbasic, H. U. Marschall, G. Zollner, P. Fickert, C. Langner, K. Zatloukal, H. Denk, and M. Trauner. 2005. CAR and PXR agonists stimulate hepatic bile acid and bilirubin detoxification and elimination pathways in mice. *Hepatology.* **42:** 420–430.
15. Fickert, P., G. Zollner, A. Fuchsbichler, C. Stumptner, C. Pojer, R. Zenz, F. Lammert, B. Stieger, P. J. Meier, K. Zatloukal, et al. 2001. Effects of ursodeoxycholic and cholic acid feeding on hepatocellular transporter expression in mouse liver. *Gastroenterology.* **121:** 170–183.
16. Lee, J., F. Azzaroli, L. Wang, C. J. Soroka, A. Gigliozzi, K. D. Setchell, W. Kramer, and J. L. Boyer. 2001. Adaptive regulation of bile salt transporters in kidney and liver in obstructive cholestasis in the rat. *Gastroenterology.* **121:** 1473–1484.
17. Kitada, H., M. Miyata, T. Nakamura, A. Tozawa, W. Honma, M. Shimada, K. Nagata, C. J. Sinal, G. L. Guo, F. J. Gonzalez, et al. 2003. Protective role of hydroxysteroid sulfotransferase in lithocholic acid-induced liver toxicity. *J. Biol. Chem.* **278:** 17838–17844.
18. Staudinger, J. L., B. Goodwin, S. A. Jones, D. Hawkins-Brown, K. I. MacKenzie, A. LaTour, Y. Liu, C. D. Klaassen, K. K. Brown, J. Reinhard, et al. 2001. The nuclear receptor PXR is a lithocholic acid sensor that protects against liver toxicity. *Proc. Natl. Acad. Sci. USA.* **98:** 3369–3374.
19. Xie, W., A. Radominska-Pandya, Y. Shi, C. M. Simon, M. C. Nelson, E. S. Ong, D. J. Waxman, and R. M. Evans. 2001. An essential role for nuclear receptors SXR/PXR in detoxification of cholestatic bile acids. *Proc. Natl. Acad. Sci. USA.* **98:** 3375–3380.
20. Schuetz, E. G., S. Strom, K. Yasuda, V. Lecureur, M. Assem, C. Brimer, J. Lamba, B. B. Kim, V. Ramachandran, B. J. Komoroski, et al. 2001. Disrupted bile acid homeostasis reveals an unexpected interaction among nuclear hormone receptors, transporters, and cytochrome P450. *J. Biol. Chem.* **276:** 39411–39418.
21. Bodin, K., U. Lindbom, and U. Diczfalusy. 2005. Novel pathways of bile acid metabolism involving CYP3A4. *Biochim. Biophys. Acta.* **1687:** 84–93.
22. Furster, C., and K. Wikvall. 1999. Identification of CYP3A4 as the major enzyme responsible for 25-hydroxylation of 5beta-cholestane-3alpha,7alpha,12alpha-triol in human liver microsomes. *Biochim. Biophys. Acta.* **1437:** 46–52.
23. Stedman, C., G. Robertson, S. Coulter, and C. Liddle. 2004. Feed-forward regulation of bile acid detoxification by CYP3A4: studies in humanized transgenic mice. *J. Biol. Chem.* **279:** 11336–11343.
24. Danielsson, H., and J. Sjovall. 1975. Bile acid metabolism. *Annu. Rev. Biochem.* **44:** 233–253.

25. Bremmelgaard, A., and J. Sjovall. 1979. Bile acid profiles in urine of patients with liver diseases. *Eur. J. Clin. Invest.* **9:** 341–348.
26. Thomassen, P. A. 1979. Urinary bile acids in late pregnancy and in recurrent cholestasis of pregnancy. *Eur. J. Clin. Invest.* **9:** 425–432.
27. Stiehl, A., R. Raedsch, G. Rudolph, U. Gundert-Remy, and M. Senn. 1985. Biliary and urinary excretion of sulfated, glucuronidated and tetrahydroxylated bile acids in cirrhotic patients. *Hepatology.* **5:** 492–495.
28. Nemeth, A., and B. Strandvik. 1984. Urinary excretion of tetrahydroxylated bile acids in children with alpha 1-antitrypsin deficiency and neonatal cholestasis. *Scand. J. Clin. Lab. Invest.* **44:** 387–392.
29. Marschall, H. U., H. Matern, B. Egestad, S. Matern, and J. Sjovall. 1987. 6 Alpha-glucuronidation of hyodeoxycholic acid by human liver, kidney and small bowel microsomes. *Biochim. Biophys. Acta.* **921:** 392–397.
30. Wang, R., P. Lam, L. Liu, D. Forrest, I. M. Yousef, D. Mignault, M. J. Phillips, and V. Ling. 2003. Severe cholestasis induced by cholic acid feeding in knockout mice of sister of P-glycoprotein. *Hepatology.* **38:** 1489–1499.
31. Perwaiz, S., D. Forrest, D. Mignault, B. Tuchweber, M. J. Phillip, P. Wang, V. Ling, and I. M. Yousef. 2003. Appearance of atypical 3alpha,6beta,7beta,12alpha-tetrahydroxy-5beta-cholan-24-oic acid in spgp knockout mice. *J. Lipid Res.* **44:** 494–502.