Removal of the bile acid pool upregulates cholesterol 7α-hydroxylase by deactivating FXR in rabbits

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Abstract **We investigated the role of the orphan nuclear receptor farnesoid X receptor (FXR) in the regulation of cholesterol 7α-hydroxylase (CYP7A1), using an in vivo rabbit model, in which the bile acid pool, which includes high affinity ligands for FXR, was eliminated. After 7 days of bile drainage, the enterohepatic bile acid pool, in both New Zealand White and Watanabe heritable hyperlipidemic rabbits, was depleted. CYP7A1 activity and mRNA levels increased while FXR was deactivated as indicated by reduced FXR protein and changes in the expression of target genes that served as surrogate markers of FXR activation in the liver and ileum, respectively. Hepatic bile salt export pump mRNA levels and ileal bile acid-binding protein decreased while sterol 12α-hydroxylase and sodium/taurocholate cotransporting polypeptide mRNA levels increased in the liver. In addition, hepatic FXR mRNA levels decreased significantly. The data, taken together, indicate that FXR was deactivated when the bile acid pool was depleted such that CYP7A1 was upregulated. Further, lack of the high affinity ligand supply was associated with downregulation of hepatic FXR mRNA levels.**—Xu, G., L-x. Pan, S. K. Erickson, B. M. Forman, B. L. Shneider, M. Ananthanarayanan, X. Li, S. Shefer, N. Balasubramanian, L. Ma, H. Asaoka, S. R. Lear, L. B. Nguyen, I. Dussault, F. J. Suchy, G. S. Tint, and G. Salen. **Removal of the bile acid pool upregulates cholesterol 7α-hydroxylase by deactivating FXR in rabbits.** ***J. Lipid Res.*** **2002.** 43: **45–50.**

Supplementary key words bile salt export pump • biosynthesis • farnesoid X receptor • ileal bile acid-binding protein • orphan nuclear receptor

Farnesoid X receptor (FXR), one of the members of the orphan nuclear receptor family expressed predominantly in the liver, kidney, intestine, and adrenal gland (1), has been identified as a ligand-activated negative regulator for the transcription of cholesterol 7α-hydroxylase (CYP7A1) (2–4), the rate-controlling enzyme for classic bile acid synthesis. The most potent in vitro ligands for FXR are the hydrophobic bile acids: chenodeoxycholic, deoxycholic, and lithocholic acids (2–4). Wang et al. (4) reported that after cotransfection with the bile acid transporters, ileal apical sodium-dependent bile acid transporter or sodium/taurocholate-cotransporting polypeptide (NTCP), free cholic acid, and all conjugated bile acids became as powerful ligands as free chenodeoxycholic acid for FXR. Furthermore, to respond to bile acids (ligands), FXR must heterodimerize with the retinoid X receptor (RXR) (2, 4).

Makishima et al. (2) reported that when associated with its ligand, FXR suppressed transcription of the gene encoding CYP7A1, but coordinately activated the gene encoding ileal bile acid-binding protein (IBABP), also known as ileal lipid-binding protein. Although the specific function of IBABP in bile acid metabolism has not been ascertained, it has been established as a target gene for activated FXR in the ileum (2, 5, 6).

Bile salt export pump (BSEP) (7) is an ATP-binding cassette-type membrane transporter that is located in canalicular microvilli of hepatocytes and is responsible for excretion of conjugated bile salts into canalicular bile (8). Ananthanarayanan et al. (9) reported that in transfected HepG2 cells, BSEP promoter activity was induced by activated FXR and suggested that BSEP also is a target gene for FXR in the liver. The full-length cDNAs of rabbit and mouse hepatic *BSEP* have been cloned by Ananthanaray-

Abbreviations: BSEP, bile salt export pump; CYP27, cholesterol 27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; FXR, farnesoid X receptor; IBABP, ileal bile acid-binding protein; NTCP, sodium/taurocholate-cotransporting polypeptide; RXR, retinoid X receptor.

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anan et al. (9) (GenBank accession no. AF249879) and Green, Hoda, and Ward (10), respectively.

NTCP is the major bile acid transporter located in the sinusoidal membrane of hepatocytes. It recovers bile acids from the portal circulation by an active process (11). It is suggested that transcription of *NTCP* is negatively regulated by FXR (12, 13).

Sinal et al. (12) demonstrated that in $FXR^{-/-}$ (*FXR* null) mice, the inhibitory effect of bile acids on CYP7A1 did not occur, supporting a regulatory role for FXR in the transcription of *CYP7A1*. In addition, their work confirmed that bile acids positively regulated transcription of *Bsep*, and sterol 12α-hydroxylase (*Cyp8b*) in the liver and *Ibabp* in the ileum in an FXR-dependent manner and that FXR was involved in negative control of *Ntcp* expression.

In this study, we investigated the role of FXR in the regulation of *CYP7A1* in vivo and established the importance of the bile acid pool in both *FXR* activation and control of hepatic mRNA levels. A bile fistula model was utilized in rabbits, in which CYP7A1 responds oppositely to dietary cholesterol as seen in rats (14) and mice (15). Abundance of hepatic BSEP, sterol 12α-hydroxylase (CYP8B), and NTCP mRNAs and intestinal IBABP protein were measured and served as surrogate markers for *FXR* activation. This in vivo model enabled us to test FXR function when the bile acid (ligand) flux through the liver was interrupted and to examine the mechanism(s) for upregulation of *CYP7A1* in vivo. We observed that in rabbits with a depleted bile acid pool, *FXR* was deactivated as indicated by reduced FXR protein and the responses of its target genes. These results suggested that upregulation of *CYP7A1* was due to removal of the inhibitory control from *FXR*. In addition, in the absence of the enterohepatic bile acid pool, FXR mRNA levels were decreased substantially, suggesting that *FXR* transcription was downregulated in response to a diminished ligand supply.

MATERIALS AND METHODS

Animal experiments

Male New Zealand White (NZW) (n = 12) and male Watanabe heritable hyperlipidemic (WHHL) (n = 12) rabbits weighing 2.5–2.75 kg (Convance, Denver, PA) were used in this study. All rabbits were fed regular rabbit chow (Purina Mills, St. Louis, MO). Bile fistulas were constructed in half of the rabbits (6 of 12) as described previously (16). Bile drainage was continued for 7 days to ensure the elimination of bile acids returning to the liver. After 3 days of bile drainage, cholic acid synthesis was maximally stimulated (17), whereas deoxycholic acid, an FXR high affinity ligand, totally disappeared from the bile after 5 days of bile drainage (16), indicating complete interruption of the bile acid flux through the liver. The animals were then killed. The livers were removed and portions were immediately frozen for measurements of FXR, BSEP, CYP8B, and NTCP mRNA levels, FXR protein levels, CYP7A1 activity and mRNA levels, and cholesterol 27-hydroxylase (CYP27) activity. The mucosa from the terminal ileum was harvested for measurements of IBABP protein.

The animal protocol was approved by the Subcommittee on Animal Studies at the Veterans Affairs Medical Center (East Orange, NJ) and by the Institutional Animal Care and Use Committee at the University of Medicine and Dentistry-New Jersey Medical School (Newark, NJ).

Biochemical analyses

Electrophoretic mobility shift assay for FXR protein. To prepare nuclear extracts, liver tissue from control and bile fistula rabbits was minced and homogenized with a Wheaton (Millville, NJ) Dounce homogenizer (pestle B) in lysis buffer containing 20 mM HEPES (pH 7.6), 10 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM DTT, leupeptin (10 μg/ml), aprotinin (10 μg/ml), 1 mM phenylmethylsulfonyl fluoride, 0.1% Triton X-100, and 20% glycerol. Nuclei were separated by centrifugation at 1,800 *g* for 5 min and suspended in the same kind of lysis buffer but now containing a high salt concentration (500 mM NaCl). The nuclei were broken with a homogenizer (Dounce, pestle A). After centrifugation in an Eppendorf centrifuge (Brinkmann Instruments, Westbury, NY) at 9,300 *g* for 10 min, the supernatant (nuclear extract) was collected and divided into aliquots and stored at −70°C. All the procedures were performed at 4°C.

The response element that was used as a specific FXR protein-binding probe was a double-stranded oligonucleotide containing the sequence 5′-AAGGTCAATGACCTTA-3′ and complementary strand 5′-TAAGGTCATTGACCTT-3′. The oligonucleotide sequences containing a mutation substitution of binding site for FXR were 5′-AAGAACAATGTTCTTA-3′ and 5′-TAAGAACA TTGTTCTT-3′. These probes were end labeled by T4 polynucleotide kinase and [γ-^{32}P]ATP.

Binding reaction mixture contained 2 μg of poly(dI-dC), 20 mM HEPES (pH 7.5), 1.5 mM $MgCl_2$, 1 mM DTT, 2 mM EDTA, 50 mM KCl, and 3% glycerol. Competitor [unlabeled (cold) or mutated probe] was added in a 100-fold excess and was preincubated with the extracted nuclear proteins (10 μg) on ice for 30 min before adding labeled probe. After another 1 h of incubation with labeled probe (0.06 pmol, 25,000 cpm) on ice, the reactions were analyzed by low ionic strength system electrophoresis on an 8% polyacrylamide gel in 0.375× TBE [0.33 mM Tris borate (pH 8.7)-1.0 mM EDTA]. The gel was then dried and subjected to autoradiography.

Western blot analysis (for IBABP). Homogenates of ileal mucosa were prepared as previously described (18). Protein concentrations were determined by the Bradford method (19) with bovine serum albumin as reference standard. Western blotting for IBABP was performed with 10 μg of homogenate. Proteins were separated in a 15% sodium dodecyl sulfate-polyacrylamide gel and electrotransferred onto nitrocellulose membranes. The blots were blocked overnight at 4°C with Tris-buffered saline containing 0.1% Tween and 5% nonfat dry milk and then incubated for 2 h at room temperature with rabbit anti-mouse ileal lipid-binding protein (also called IBABP) polyclonal antibody (a gift from M. W. Crossman, Washington University, St. Louis, MO). Immune complexes were detected with ^{125}I-labeled protein A. Immunoreactive bands were detected in the linear range of response with a PhosphorImager screen and quantified by using a PhosphorImager and Imagequant software (Molecular Dynamics, Sunnyvale, CA).

Northern blotting analyses. Total RNA from samples of frozen liver was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (20). The total RNA pellet was dissolved in 100 μl of diethylpyrocarbonate-treated water. Poly(A)$^+$ was isolated by oligo(dT)-cellulose chromatography (21). The relative levels of FXR, CYP7A1, BSEP, CYP8B, and NTCP mRNAs were quantitated by Northern blotting analysis as previously described by Ness, Keller, and Pendelton (22), except that glyceraldehyde-3-phosphate dehydrogenase served as the internal reference standard. The cDNA for rat *CYP7A1* was a gift from J. Y. L. Chiang (Department of Biochemistry and Molecular Pathology,

Northeastern Ohio Universities College of Medicine, Rootstown, OH), that for FXR was from R. Evans and B. M. Forman (Department of Molecular Medicine, City of Hope National Medical Center, Duarte, CA), *CYP8B* was from I. Björkhem (Karolinska Institute, Stockholm, Sweden), and the cDNAs for *BSEP* and *NTCP* were from M. Ananthanarayanan (Department of Pediatrics, Mt. Sinai School of Medicine, New York, NY).

Assays for activities of CYP7A1 and CYP27. Hepatic microsomes and mitochondria were prepared by differential ultracentrifugation (23), and protein was determined according to Lowry et al. (24). CYP7A1 activity was measured in hepatic microsomes by the isotope incorporation method of Shefer, Salen, and Batta (23). The isotope incorporation method for measurement of mitochondrial CYP27 activity was as previously described by Shefer et al. (25)

Assay for bile acids. Bile acids were analyzed by capillary gas–liquid chromatography as previously described (16).

Statistical study

Data are shown as means ± SD and were compared statistically by Student's *t*-test (unpaired). BMDP Statistical Software (Los Angeles, CA) was used for statistical evaluations.

RESULTS

The percentage of glycine-conjugated deoxycholic acid in the bile acid pool was measured in bile collected during the first 30 min after insertion of the bile fistula and comprised 85 ± 7% in NZW and 83 ± 9% in WHHL rabbits.

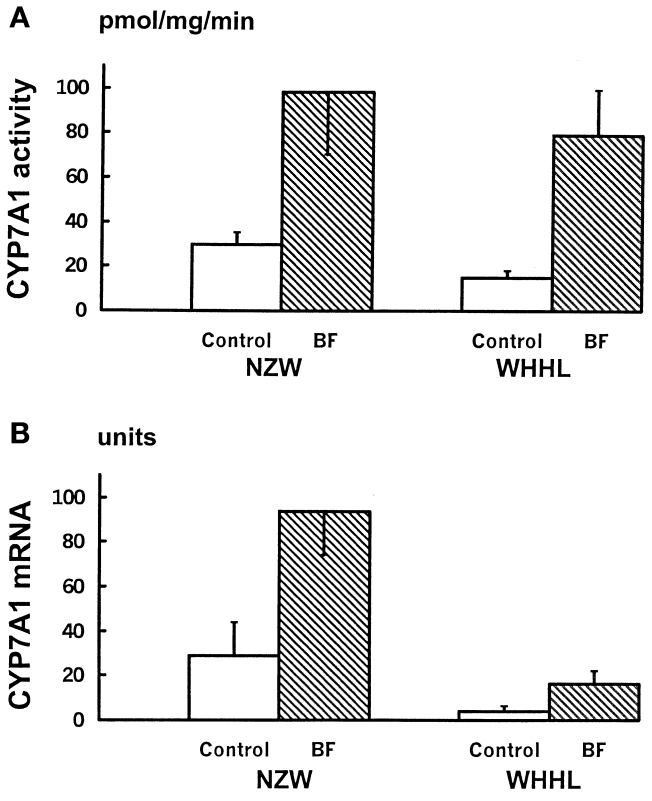

Fig. 1. CYP7A1 activity and mRNA levels in controls and after 7 days of bile fistula drainage (BF). Both CYP7A1 activity (A) and mRNA levels (B) increased significantly in NZW and WHHL rabbits after depletion of the bile acid pool.

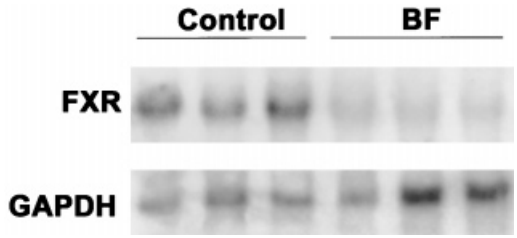

Fig. 2. Representative Northern blots for FXR mRNA. After bile fistula drainage (BF), FXR mRNA was barely detected.

Relatively small amounts of cholic acid (14–16%) and traces of chenodeoxycholic acid (1%) were also detected in these bile samples. However, after 5 days of bile drainage, glycocholic acid became the predominant bile acid (99%) in the bile and deoxycholic acid, which was formed by bacterial 7α-dehydroxylation of cholic acid in the intestine, was no longer detected, demonstrating complete interruption of the enterohepatic circulation of bile acids.

After 7 days of bile drainage, CYP7A1 activity increased 3.3 times ($P < 0.001$) from 30 ± 5 to 98 ± 28 pmol/mg per min in NZW rabbits and 5.3 times ($P < 0.0001$) from 15 ± 3 to 79 ± 20 pmol/mg per min in WHHL rabbits (**Fig. 1A**). CYP7A1 mRNA levels also rose 3.2 times from 29 ± 15 to 94 ± 20 units ($P < 0.001$) in NZW rabbits and four times from 4 ± 2 to 16 ± 6 units ($P < 0.01$) in WHHL rabbits (Fig. 1B).

CYP27 activity that reflected alternative bile acid synthesis did not change after removal of the bile acid pool in either NZW (29.0 ± 5.5 vs. 24.0 ± 9.9 pmol/mg per min) or WHHL rabbits (23.0 ± 4.5 vs. 33.8 ± 9.8 pmol/mg per min).

After depletion of the bile acid pool, FXR mRNA levels were barely detected (**Fig. 2**) and decreased 74% (from 100 ± 38 to 26 ± 4 units, $P < 0.05$) in NZW rabbits and 56% (100 ± 31 to 44 ± 13 units, $P < 0.05$) in WHHL rabbits (**Fig. 3**).

Figure 4 shows FXR protein as determined by electrophoretic mobility shift assays (EMSA) in control rabbits (C) and rabbits after bile fistula bile drainage. The specificity of the binding complex was evaluated by adding an

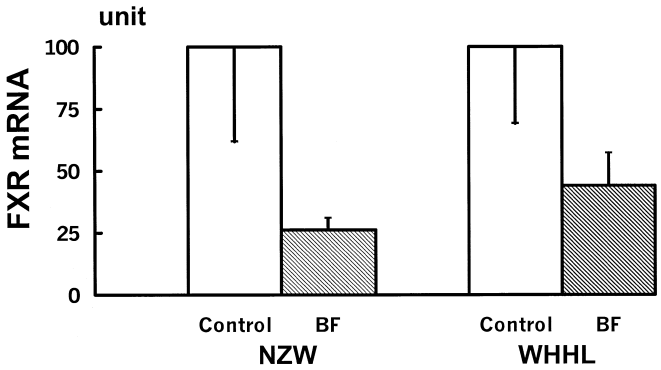

Fig. 3. Densitometric measurements of FXR mRNA after removal of the bile acid pool. FXR mRNA levels declined significantly in both NZW and WHHL rabbits after bile fistula drainage (BF).

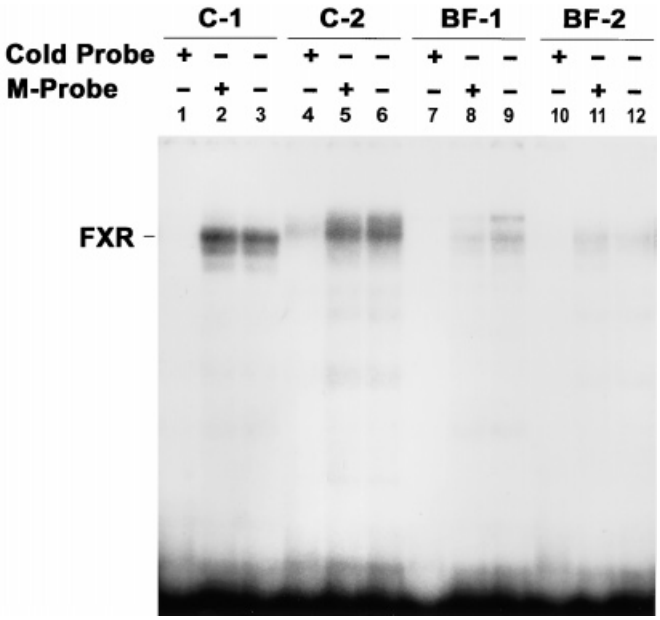

Fig. 4. Measurement of hepatic FXR protein by electrophoretic mobility shift assay. The specificity of the FXR-RXR complexes was evaluated with an excess of unlabeled FXR response element (cold probe) or mutated FXR response element (M-probe) as competitor. Specific binding was identified by addition of a 100-fold excess of the unlabeled FXR response element that made the specific binding complexes not visualized (lanes 1, 4, 7, and 10). A mutated FXR response element (M-probe) failed to act as a competitor (lanes 2, 5, 8, and 11), suggesting that the observed complex is specific. The results suggested that FXR protein levels decreased after bile acid depletion as the specific binding was abundant in controls (lanes 3 and 6) but was barely visualized after bile fistula (BF) (lanes 9 and 12).

excess of either unlabeled FXR response element (cold probe) or mutated FXR response element (M-probe) as competitor. When excess unlabeled (cold) probe was added, the specific binding of the labeled probe to FXR protein was limited and not visualized (Fig. 4, lanes 1, 4, 7, and 10). Adding an excess of the unlabeled mutated response element (M-probe) did not interfere with the specific binding of the labeled probe to FXR protein (Fig. 4, lanes 2, 5, 8, and 11). In Fig. 4, abundant specific binding of the labeled probe to FXR was noted in controls (Fig. 4, lanes 3 and 6) but barely visualized in rabbits after bile fistula (Fig. 4, lanes 9 and 12). Densitometric measurements revealed that FXR protein decreased 47% ($P < 0.01$), from a baseline of 52.1 ± 8.9 units (n = 4) to 27.7 ± 8.4 units (n = 4) in NZW rabbits after bile drainage for 7 days. Thus, removal of the bile acid pool produced a significant decrease of FXR protein.

The activation status of FXR was evaluated by determining changes in the expression of key target genes for FXR in control and bile fistula rabbits. IBABP served as a marker of FXR activation in the ileum, and was determined by Western blotting analysis. **Figure 5** shows that in controls (lanes 1, 3, 5, 7, and 9), the mass of IBABP is higher in intensity than in rabbits after bile fistula (lanes 2, 4, 6, 8, and 10). The protein levels decreased 57% (1.00 ± 0.12 to 0.43 ± 0.07 unit, $P < 0.001$) in NZW rabbits and 56% (3.42 ± 0.71 to 1.49 ± 0.73 units, $P < 0.01$) in WHHL rabbits (**Fig. 6**) after bile fistula. Similarly, the mRNA levels of BSEP, which serves as a marker of FXR activation in the liver, declined 58% (from 45.4 ± 6.4 to 19.1 ± 4.0 units, $P < 0.001$) in NZW rabbits, and 25% (from 40.0 ± 4.2 to 30.1 ± 3.8 units, $P < 0.05$) in WHHL rabbits (**Fig. 7**). In contrast, mRNA levels of hepatic CYP8B and NTCP, which are negatively regulated by FXR, increased more than 2-fold (from 0.089 ± 0.013 to 0.200 ± 0.030 unit, $P < 0.01$) and more than three times (from 0.06 ± 0.02 to 0.19 ± 0.05 unit, $P < 0.01$), respectively, in NZW rabbits after bile acid depletion (**Fig. 8**). Northern blots for these target genes of FXR in control and bile fistula NZW rabbits are shown in **Fig. 9**.

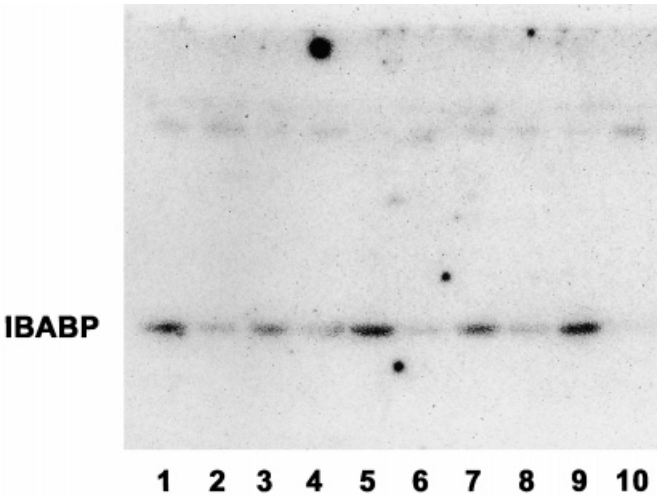

Fig. 5. Western blots for IBABP in control rabbits (lanes 1, 3, 5, 7, and 9) and bile fistula rabbits (lanes 2, 4, 6, 8, and 10). The mass of IBABP binding was higher in controls than in rabbits after bile drainage.

DISCUSSION

This study was designed to evaluate the role of the orphan nuclear receptor, FXR, in the regulation of CYP7A1

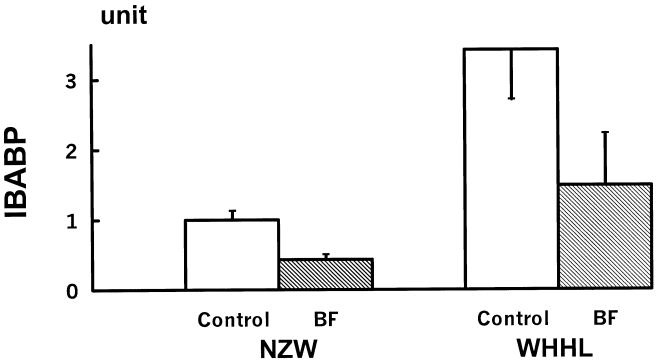

Fig. 6. Densitometric measurements of IBABP after removal of the bile acid pool. IBABP levels in the ileum decreased significantly in NZW and WHHL rabbits after bile fistula drainage (BF).

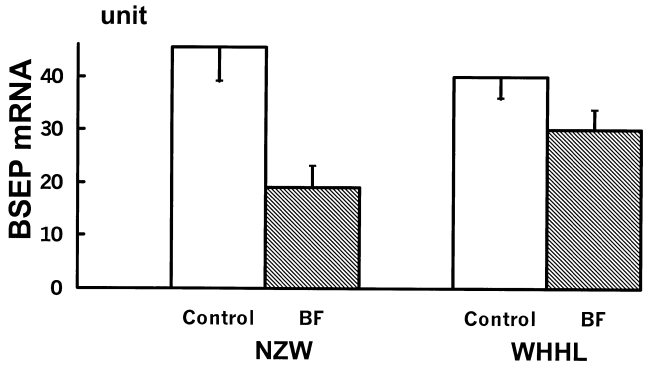

Fig. 7. Densitometric measurements of BSEP mRNA levels after removal of the bile acid pool. BSEP mRNA levels decreased significantly in NZW and WHHL rabbits after bile fistula drainage (BF).

in an in vivo whole rabbit model. Specifically, our aim was to elucidate the effect of the absent ligand flux (bile acids) returning to the liver on FXR as reflected by activated FXR protein and changes in the transcription of both positively and negatively regulated FXR target genes. We demonstrated that removal of the bile acid pool diminished the expression of BSEP and IBABP, which coincided with the upregulation of CYP7A1, NTCP, and CYP8B. Most importantly, measurements of FXR protein by EMSA showed a significant reduction (−47%) after bile drainage. Because FXR must first form a heterodimer with RXR (2, 4) before it can be activated by ligands (bile acids) and bind to the appropriate response element, the FXR protein measured by EMSA was actually in the form of FXR/RXR. These results demonstrated the inverse relationship between CYP7A1 and FXR in the liver such that removal of the bile acid (ligands) pool deactivated FXR and released *CYP7A1* transcription from the inhibitory control of FXR.

A second major finding was that FXR mRNA levels diminished markedly after the bile acid ligand supply for FXR was eliminated, consistent with the aforementioned finding that FXR protein levels measured by EMSA decreased significantly in rabbits after bile acid depletion.

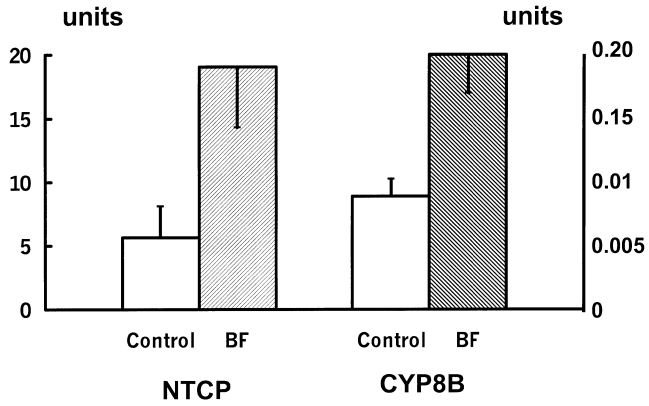

Fig. 8. mRNA levels of NTCP and sterol 12α-hydroxlase (CYP8B) in New Zealand White rabbits increased ($P < 0.01$) after bile fistula drainage (BF).

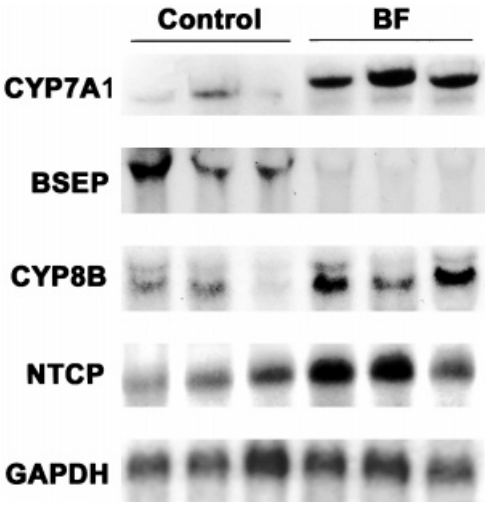

Fig. 9. Representative Northern blots for mRNA levels of CYP7A1, BSEP, sterol 12α-hydroxylase (CYP8B), and NTCP in control and bile acid-depleted NZW rabbits (BF). The expression of mRNA for CYP7A1, CYP8B, and NTCP, which are negatively regulated by FXR, increased whereas BSEP mRNA, which is positively regulated by FXR, decreased. GAPDH, Glyceraldehyde-3-phosphate dehydrogenase.

Taken together, these results suggest that the hepatic bile acid flux not only supplies ligands for the activation of FXR but also may be involved in the transcription of *FXR*.

In contrast to studies of the *FXR* gene knockout mouse model (12), we studied the role of FXR in the regulation of *CYP7A1* and other key target genes in an in vivo whole rabbit model with normal genes, where FXR was deactivated by the total elimination of the ligand supply. Moreover, bile acid metabolism in rabbits is different from rodents, where deoxycholic acid, a hydrophobic bile acid, predominates (85%) in the enterohepatic pool. Thus, our study of rabbits can be viewed as complementary to the report of Sinal et al. (12) on *FXR* null mice.

Until the present, no method was available for the direct measurement of hepatic FXR activity in an in vivo animal model. Thus, in this study, we used IBABP protein levels in the ileum and BSEP, CYP8B, and NTCP mRNA levels in the liver to serve as surrogate markers that would indicate the activation status of FXR in the ileum and the liver, respectively. After 7 days of bile drainage, the bile acid flux returning to the liver was eliminated as evidenced by complete disappearance of deoxycholic acid from hepatic bile. Significant reductions of IBABP protein and BSEP mRNA levels coupled with increased CYP8B and NTCP mRNA levels occurred and strongly indicated that FXR was deactivated by the depletion of the bile acid flux. It was noteworthy that although IBABP is located in the ileum and BSEP, CYP8B, NTCP, and CYP7A1 are expressed in the liver, absence of the circulating bile acid pool from the gut to the liver produced the expected changes in these FXR target genes. Under these conditions, the activation status of FXR in the ileum reflected by IBABP mirrored FXR activation in the liver.

We also used WHHL rabbits in these experiments. In

WHHL rabbits, where LDL receptors are deficient and baseline CYP7A1 activity and mRNA levels are lower than NZW rabbits (26), removal of bile acid pool also deactivated FXR and downregulated transcription.

It was also noteworthy that CYP27 activity was not affected by depletion of the bile acid pool, and by inference, was not altered by changes in FXR activation or transcription. Thus, in rabbits, alternative bile acid synthesis initiated by CYP27 does not appear to be regulated by FXR. This finding agrees with the negative results for *CYP27* transcription in *FXR* null mice reported by Sinal et al. (12).

In summary, FXR protein was measured by EMSA and activation status of FXR was assessed by changes in mRNA levels of key FXR target genes *BSEP*, *CYP8B*, and *NTCP* in the liver and by protein levels of IBABP in the ileum. In an in vivo whole rabbit model, removal of the bile acid pool diminished and deactivated FXR protein. Thus, eliminating the hepatic bile acid flux not only resulted in upregulation of CYP7A1 by removing the inhibitory effect of FXR, but also decreased mRNA levels of FXR, suggesting the possibility that the ligand flux might also be involved in the control of *FXR* transcription.

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