Regulation of the Farnesoid X Receptor (FXR) by Bile Acid Flux in Rabbits*

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We investigated the roles of hydrophobic deoxycholic acid (DCA) and hydrophilic ursodeoxycholic acid (UCM) in the regulation of the orphan nuclear farnesoid X receptor (FXR) in vivo. Rabbits with bile fistula drainage (removal of the endogenous bile acid pool), rabbits with bile fistula drainage and replacement with either DCA or UCM, and intact rabbits fed 0.5% cholic acid (CA) (enlarged endogenous bile acid pool) were studied. After bile fistula drainage, cholesterol 7α-hydroxylase (CYP7A1) mRNA and activity levels increased, FXR-mediated transcription was decreased, and FXR mRNA and nuclear protein levels declined. Replacing the enterohepatic bile acid pool with DCA restored FXR mRNA and nuclear protein levels and activated FXR-mediated transcription as evidenced by the increased expression of its target genes, SHP and BSEP, and decreased CYP7A1 mRNA level and activity. Replacing the bile acid pool with UCM also restored FXR mRNA and nuclear protein levels but did not activate FXR-mediated transcription, because the SHP mRNA level and CYP7A1 mRNA level and activity were unchanged. Feeding CA to intact rabbits expanded the bile acid pool enriched with the FXR high affinity ligand, DCA. FXR-mediated transcription became activated as shown by increased SHP and BSEP mRNA levels and decreased CYP7A1 mRNA level and activity but did not change FXR mRNA or nuclear protein levels. Thus, both hydrophobic and hydrophilic bile acids are effective in maintaining FXR mRNA and nuclear protein levels. However, the activating ligand (DCA) in the enterohepatic flux is necessary for FXR-mediated transcriptional regulation, which leads to down-regulation of CYP7A1.

The farnesoid X receptor (FXR) is an orphan nuclear transcription factor that has recently been identified as a negative regulator of CYP7A1, a gene encoding cholesterol 7α-hydroxylase, the rate-limiting enzyme in the classic bile acid synthesis pathway (1–3). Based on in vitro studies in cell culture, the most effective (high affinity) ligands for FXR activation are chenodeoxycholic acid (CDCA), deoxycholic acid (DCA), and lithocholic acid (LA), which are all hydrophilic bile acids. In contrast, hydrophilic bile acids such as ursodeoxycholic acid and muricholic acid are not effective. Wang et al. (1) reported that in CV-1 cells co-transfected with bile acid transporters, free cholic acid (CA), and the glycine or taurine conjugates of CA, DCA, and LA, which now are hydrophilic, became strong activators of FXR comparable with free CDCA.

Recently, other target genes for FXR have been identified that are positively regulated, including the bile salt export pump (BSEP), which is responsible for the canalicular transport of bile acids (4), and short heterodimer partner (SHP), which plays an important role in the feedback regulation of CYP7A1 by bile acids (5, 6). FXR/RXR does not bind directly to the bile acid response element in the promoter region of human CYP7A1 (7); thus, CYP7A1 transcriptional down-regulation by bile acids via FXR is indirect via SHP (5, 6). The increased SHP is believed to inactivate liver receptor homolog-1 (LRH-1), an essential transcription factor for CYP7A1 expression (8). Recently, Chen et al. (9) suggested that fetoprotein transcription factor (FTF), a human homolog of mouse LRH-1, was an inhibitor rather than a transcription factor for CYP7A1. More recently, studies in SHP knock-out mice (10, 11) suggested that CYP7A1 could also be regulated by bile acids through SHP-independent pathways, because cholic acid feeding to the SHP−/− mice also repressed CYP7A1 expression. Therefore, the mechanism by which activated FXR down-regulates CYP7A1 has not been completely elucidated.

To examine the proposed theories of the role of bile acids in FXR activation and the regulation of CYP7A1 under in vivo conditions, this study was carried out in rabbits with depleted bile acid pool/flux, which was replaced with either hydrophobic DCA or hydrophilic ursodeoxycholic acid (UCM), and intact rabbits

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Experimental Procedures

Experimental Design—Male New Zealand White (NZW) (n = 24) rabbits weighing 2.5–2.75 kg (Convance, Denver, PA) were used in this study. Sixteen rabbits were fed regular rabbit chow, and eight rabbits were fed regular chow containing 0.5% CA (Purina Mills, St. Louis, MO) for 7 days. Bile fistulas were constructed in 12/16 regular chow-fed rabbits and 4/8/0.5% CA chow-fed rabbits as described previously (12). Bile drainage was continued for 7 days to ensure the complete elimination of bile acids returning to the liver. The secondary bile acid, DCA, a high-affinity ligand for FXR, totally disappeared from the hepatic bile after 5 days of bile drainage, indicating the interruption of the intestinal bile acid flux through the liver. Four of the twelve rabbits with 7 days of bile fistula drainage were sacrificed to collect liver specimens, which were immediately frozen for measurements of FXR, SHP, BSEP, and FTF mRNA levels, FXR/RXR and FTF nuclear protein levels, and CYP7A1 mRNA levels and cholesterol 7α-hydroxylase activity. The sodium salts of glyco-DCA and glyco-UCa were dissolved in water. In the bile acid-depleted rabbits, after bile acid synthesis was maximally stimulated and bile acid output became constant, glyco-DCA (n = 4) and glyco-UCa (n = 4) were then infused intraduodenally. The baseline bile acid flux was determined by measuring the bile acid output for 30 min immediately after construction of the bile fistula. To ensure sufficient hepatic bile acid replacement, glyco-DCA or glyco-UCa were infused at a rate of 60 mg (conjugated bile acid) per hour for 24 h. After completion of the 24 h infusions, including cholesynthetic controls and 5% CA-fed controls, bile was reinfused, and liver tissues were separated by centrifugation at 1800 × g for 10 min before adding the 32P-labeled probe. After a 1-h incubation with the labeled probe (0.06 μg), the reactions were analyzed by electrophoresis at 1800 °C to separate the radiolabeled DNA. The response element used as the complementary strand. These probes were end-labeled by a TdT Quick coupled transcription kit (Amersham Pharmacia Biotech, Piscataway, NJ). The sequences of the mutant probe were 5′-AGGC-3′ and 5′-AAGGTCAATGA-3′, respectively.

RESULTS

Bile Fistula Drainage—As described previously (12), after 7 days of bile fistula drainage the secondary bile acid, DCA, disappeared from the hepatic bile, which indicated that the enterohepatic bile acid pool/flux was totally depleted and that no further intestinal bile acids had returned to the liver. Removal of the bile acid pool/flux increased CYP7A1 mRNA levels 2.3-fold and activity 5.7-fold (p < 0.001), respectively (Fig. 1). FXR/RXR nuclear protein levels measured by gel shift assays (Fig. 2) were reduced 65% (p < 0.01), and FXR mRNA levels (Figs. 3 and 4) decreased 41% (p < 0.05). The levels of FXR target genes SHP and BSEP mRNAs decreased 82% (p < 0.01) and 58% (p < 0.05), respectively (Figs. 5 and 6). However, there was no change in FXR nuclear protein (Fig. 7) or mRNA levels (Fig. 8).

Replacing the Bile Acid Flux with either DCA or UCA in Bile Acid-depleted Rabbits—Infusing sodium glyco-DCA intraduodenally for 24 h restored the hepatic bile acid pool/flux to 49 ± 8 mg/h, of which 88% was DCA. At baseline, the hepatic bile acid pool/flux in these rabbits measured within the first half-hour immediately after bile fistula drainage was 30 ± 10 mg/h with 85% DCA and 13% CA. After replacement with sodium glyco-UCA, the bile acid pool/flux was restored to 46 ± 8 mg/h, of which 82% was UCA (38 ± 8 mg/h) with 17% CA and 6.0%...
Replacement with DCA decreased CYP7A1 mRNA 54% \((p < 0.01)\) and activity 55% \((p < 0.01)\), whereas replacing the flux with UCA had no effect (Fig. 1). FXR mRNA levels and FXR/RXR nuclear protein increased significantly and recovered to baseline levels after the bile acid flux was restored with either DCA (hydrophobic) or UCA (hydrophilic) for 24 h (Figs. 2 and 3). SHP and BSEP mRNA levels increased 3.7-fold \((p < 0.001)\) and 2-fold \((p < 0.05)\), respectively, 24 h after replacement with DCA as compared with the low levels in rabbits with DCA. Replacement with DCA decreased CYP7A1 mRNA 54% \((p < 0.01)\) and activity 55% \((p < 0.01)\), whereas replacing the flux with UCA had no effect (Fig. 1).
depleted bile acid pools (Figs. 5 and 6). In contrast, when the hepatic bile acid pool/flux was reestablished with UCA, there was no change in SHP mRNA level but a 49% rise in BSEP mRNA (Figs. 5 and 6), but FTF nuclear protein and mRNA levels remained unchanged (Figs. 7 and 8).

**Relationship between SHP and CYP7A1 mRNA Expression**—The relationship between SHP and CYP7A1 mRNA expression is described by the curve shown in Fig. 9. The data plotted are from five groups of rabbits (three rabbits in each group) under different treatments, i.e., controls, rabbits after 7 days of bile fistula drainage where the bile acid pool was depleted, rabbits after the depleted bile acid pool was replaced with DCA or UCA for 24 h, and rabbits with intact bile acid enterohepatic circulation fed 0.5% CA for 7 days. Mathematical analysis showed that the relationship between SHP and CYP7A1 mRNA expression can be described satisfactorily by the equation CYP7A1 = 0.180 − 0.181 ln(SHP) (r² = 0.915, p < 0.0001) where CYP7A1 and SHP represent the relative units of mRNA abundance, respectively.

**DISCUSSION**

The leading theories concerning the mechanism of FXR activation by bile acids and the role of activated FXR in feedback regulation of CYP7A1 were based on findings derived from *in vitro* studies in cultured cells. Therefore, it became necessary to investigate these mechanisms in an *in vivo* whole animal model. In this study, we attempted to clarify: 1) the critical role of the FXR-activating ligand (DCA) in the enterohepatic bile acid flux for FXR activation; 2) whether bile acids play any role in the regulation of FXR transcription aside from a ligand role; and 3) whether FXR is the dominant regulator for bile acid synthesis (CYP7A1) and the canalicular transporter (BSEP).

This study demonstrated that in the *in vivo* bile acid-depleted rabbit model, replacing the enterohepatic fluid with DCA effectively activated FXR and regulated its downstream target genes. As we reported previously (19), when the endogenous bile acid pool consisting of 85% DCA was totally removed by bile fistula drainage for 7 days, CYP7A1 mRNA and activity levels increased significantly, because FXR transcriptional activity was muted as indicated by the decreased expression of FXR target genes. Importantly, the present work shows that after restoration of the enterohepatic bile acid pool/flux with DCA, FXR target gene expression also was restored as indicated by the increase in the expression of SHP and BSEP.
mRNAs and the decrease in CYP7A1 mRNA and activity relative to rabbits with depleted bile acid pools. When the enterohepatic bile acid pool/flux, which contained 88% DCA, was increased 2-fold by feeding 0.5% CA to rabbits with an intact enterohepatic circulation, CYP7A1 mRNA and activity values were significantly lower than the control values because FXR was activated as indicated by the increased mRNA levels of SHP and BSEP as compared with controls. Thus, these results consistently demonstrated that DCA was an effective FXR agonist in vivo.

The inverse relationship between SHP and CYP7A1 mRNA expression is shown by the curve in Fig. 9 and described by the equation CYP7A1 = 0.180 – 0.181 ln(SHP). The data in this figure were plotted from five groups of rabbits with different treatments (controls, bile fistula drainage, DCA and UCA replacement, and CA feeding). The expression levels of SHP and CYP7A1 in the curve shown in Fig. 9 represent a significant inverse relationship (r² = 0.915, p < 0.0001). Mathematical analysis suggests that the effect of SHP on CYP7A1 is independent and saturable. When SHP expression is low (limited supply of activating ligand for FXR), CYP7A1 is elevated and sensitive to the increase in SHP expression, although when SHP expression rises beyond certain high levels, the increase in SHP mRNA does not result in further significant repression of CYP7A1. Although recent studies in Shp knock-out mice (10, 11) demonstrated that other pathways were involved in the down-regulation of CYP7A1, these studies strongly indicated that activated FXR repressed CYP7A1 via SHP. The results of this study in the rabbit model support the hypothesis that activation of FXR induces SHP expression that down-regulates CYP7A1 transcription.

However, when the depleted bile acid pool was restored with UCA, there was little effect on CYP7A1 mRNA and activity or FXR activation as indicated by the lack of effect on the SHP mRNA level. However, after UCA replacement, the FXR nuclear protein was restored to baseline levels, although down-stream FXR-dependent transcriptional activity apparently was not. This mirrors the in vitro studies on the high affinity ligands for FXR (1), which show that hydrophilic, ursodeoxycholic acid is not an activating ligand of FXR.

This study further demonstrated that the activation of FXR and its target genes was not solely dependent on changes in the size of the bile acid pool and enterohepatic flux per se. Infusing UCA restored the enterohepatic bile acid pool/flux but did not activate FXR, because the composition of the bile acid flux/pool was changed to 82% UCA with only 17% CA and 0.6% DCA. The amounts of CA and/or DCA were apparently below the ligand-activating threshold for FXR. Therefore, the composition of the circulating bile acid pool plays a critical role in determining the nuclear activation state of FXR. In other words, a sufficient enterohepatic circulating flux of activating/high affinity ligands is required for the activation of FXR and its subsequent effects on bile acid metabolism. Previously, we noted that in cholesterol-fed rabbits the expanded bile acid pool was the determining factor for the regulation of CYP7A1 (12). Now we better understand that in cholesterol-fed rabbits, >85% of the expanded bile acid pool was the FXR high affinity activating ligand, DCA. Not only did the bile acid pool double in size with cholesterol feeding, but the availability of a high affinity ligand supply of DCA also increased, resulting in the activation of FXR with the subsequent down-regulation of CYP7A1. It is interesting to note that in rabbits with depleted bile acid pool, a 24-h infusion of glyco-DCA at the same hepatic bile acid flux as controls did not completely restore SHP mRNA to the baseline levels seen in the controls. These results indicate that factors other than bile acid pool composition and hepatic flux might also affect the expression of SHP, and these non-bile acid factors might also be induced by bile fistula drainage.

An important new observation in this study is that bile acids also regulate FXR mRNA levels. After the enterohepatic bile acid pool was removed, FXR mRNA and nuclear protein levels decreased significantly. When the depleted bile acid flux was restored with either DCA or UCA, the FXR mRNA and nuclear protein levels recovered to baseline values in controls without bile fistula. This suggests that bile acids, including those that are not activating/high affinity ligands of FXR, are necessary for maintenance of FXR mRNA and nuclear protein levels. As was observed in CA fed mice (20), FXR mRNA levels were not increased. This suggests that increasing the bile acid pool beyond normal control levels has no effect on FXR mRNA. However, the bile fistula/bile acid replacement experiments described here demonstrate that a minimum level of bile acids in the enterohepatic circulation is required for the maintenance of FXR mRNA and nuclear protein levels at baseline values in the intact controls. The mechanisms of this regulation are still unknown.

In this study, we did not find significant changes in FTF protein and mRNA levels in rabbits after removal of the bile acid pool where FXR was deactivated and CYP7A1 was up-regulated or in CA fed rabbits where the bile acid pool doubled, FXR was activated, and CYP7A1 was down-regulated. Therefore, changes in the activation of FXR or the regulation of CYP7A1 are not necessarily reflected by changes in the FTF protein or mRNA in the in vivo rabbit model.

Although in vitro studies (1–3) showed that the most effective ligands for FXR are hydrophobic bile acids such as DCA, CDCA, and LA, whereas hydrophilic bile acids, such as ursodeoxycholic acid and muricholic acids are not effective ligands for FXR, the mechanism of how bile acids activate FXR is not totally understood. However, we should point out that hydrophobicity is not the sole criterion for determining whether a bile acid is an activating ligand for FXR, because hydrophobicity refers to the water insolubility of the bile acid. For example, to infuse DCA dissolved in a water solution into the duodenum, the hydrophobic DCA was conjugated with the amino acid glycine, making it hydrophilic; but replacing the bile acid pool with hydrophilic glyco-DCA also activated FXR.

Measurements of FXR activity in this study were important for understanding the effect of bile acids on the function of FXR in the regulation of CYP7A1. Presently, there is no method available for the direct measurement of FXR activation in vivo. In our studies, we measured the changes in the expression of target genes of FXR as markers for the activation of FXR. SHP mRNA levels always mirrored the changes in FXR activation. However, although BSEP mRNA levels were regulated by activated FXR, other bile acid-dependent mechanisms also appear to be involved. UCA did not activate FXR but did produce similar quantitative biliary bile acid outputs such as DCA with a 48% increase of BSEP mRNA. This suggested that in addition to FXR, BSEP might also be regulated by the bile acid flux in an FXR-independent manner.

In summary, this study demonstrated in the rabbit model that DCA is a potent ligand for FXR, activated FXR mediates negative regulation of CYP7A1, and bile acids are required to maintain FXR mRNA and nuclear FXR protein levels. Non-activating ligand bile acids can induce FXR mRNA and protein, but the FXR protein is not activated and cannot down-regulate CYP7A1. Therefore, activation of FXR is not solely dependent on the size of the bile acid pool but, as importantly, on the
proportion of activating ligands of FXR in the circulating bile acid pool/flux mixture.

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