Farnesoid X Receptor Responds to Bile Acids and Represses Cholesterol 7α-Hydroxylase Gene (CYP7A1) Transcription*

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Cholesterol 7α-hydroxylase gene (CYP7A1) transcription is repressed by bile acids. The goal of this study is to elucidate the mechanism of CYP7A1 transcription by bile acid-activated farnesoid X receptor (FXR) in its native promoter and cellular context and to identify FXR response elements in the gene. In Chinese hamster ovary cells transfected with retinoid X receptor (RXRα/RXRα/FXR), only chenodeoxycholic acid (CDCA) and deoxycholic acid (DCA) were able to stimulate a heterologous promoter/reporter containing an eddyson response element. In HepG2 cells, all bile acids (25 μM) were able to repress CYP7A1/luciferase reporter activity, and only CDCA and DCA further repressed reporter activity when cotransfected with RXRα/FXR. The concentration of CDCA required to inhibit 50% of reporter activity (IC50) was determined to be approximately 25 μM without FXR and 10 μM with FXR. Deletion analysis revealed that the bile acid response element located between nucleotides −148 and −128 was the FXR response element, but RXRα/FXR did not bind to this sequence. These results suggest that bile acid-activated FXR exerts its inhibitory effect on CYP7A1 transcription by an indirect mechanism, in contrast to the stimulation and binding of FXR to intestinal bile-acid-binding protein gene promoter. Results also reveal that bile acid receptors other than FXR are present in HepG2 cells.

The conversion of cholesterol to bile acids in the liver is initiated by cholesterol 7α-hydroxylase, the rate-limiting enzyme in bile acid biosynthesis pathway (1). Transcription from the CYP7A1, which encodes cholesterol 7α-hydroxylase, is regulated by hormones, dietary factors, and diurnal rhythm (1). The feedback repression of CYP7A1 transcription by bile acids is an important physiological mechanism for maintaining bile acid and cholesterol homeostasis. Two bile acid response elements, BARE-I and BARE-II, have been identified previously (2, 3). These DNA sequences contain AGGTCA direct repeats similar to the elements recognized by nuclear receptors, which regulate transcription of target genes in response to ligands such as steroids and thyroid hormones, retinoids, and fatty acids. A direct repeat separated by four nucleotides (DR4) in the BARE-I region (nt −75 to −54) is bound by the oxysterol receptor LXR and by COUP-TFII, the activating ligand for which is unknown (4–6). Although deletion of BARE-I did not affect bile acid responsiveness of the CYP7A1 deletion of the BARE-II (nt −149 to −118), which contains overlapping DR1 and DR5 motifs, abolished this response (3). We have shown that the nuclear receptor HNF4 and retinoid X receptor-α (RXRα) and retinoid acid receptor-α heterodimers, respectively, bind DR1 and DR5 elements in the rat CYP7A1 promoter (5). Based on these results, we hypothesized that some nuclear receptors may respond to bile acids and repress CYP7A1 expression (4, 5, 7).

It was originally shown that the orphan nuclear receptor FXR, expressed only in the liver, gut, kidney, and adrenal cortex, activates transcription in response to micromolar amounts of farnesol and its metabolites (8–10). The preferred DNA binding sequence for FXR is an inverted repeat separated by one base pair (IR1), although DR4 and DR5 motifs are also weakly bound (8, 9). FXR is a member of nuclear receptors subfamily consisting of eddyson receptor (EcR), vitamin D3 receptor, and liver orphan receptors (LXRα and β), which are most closely related to FXR. It has been suggested that FXR is involved in the feedback control of isoprenoid synthesis and cell growth (11). Recently, several laboratories have characterized bile acids as endogenous ligands for FXR (12–15). The studies presented here attempted to dissect the mechanism of transcriptional repression of the CYP7A1 by the bile acid-activated FXR.

EXPERIMENTAL PROCEDURES

Materials—Human hepatoma cell line HepG2 was obtained from American Type Culture Collection (ATCC HB8065) (Manassas, VA). Dulbecco’s modified Eagle’s medium/F-12 and trypsin-EDTA were purchased from Life Technologies, Inc. (Cleveland, OH). Penicillin G/streptomycin and fetal bovine serum were from Celox (Hopkins, MN) and Irvine Scientific (Santa Ana, CA), respectively. Bile acids and their conjugates were supplied by Sigma. Reporter lysis buffer and luciferase assay system were purchased from Promega (Madison, WI). The β-galactosidase expression plasmid pCMVβ was from CLONTECH (Palo Alto, CA). The expression plasmid for rat FXRα, pCMX-hFXRα, was a gift from Dr. R. Evans (Salk Institute, La Jolla, CA). Rat, human, and hamster CYP7A1/luciferase chimeric plasmids were constructed as described previously (3, 16–18).

Cell Culture and Transfection Assay—HepG2 cells were cultured in Dulbecco’s modified Eagle’s medium/F-12 (50:50) supplemented with 10% (v/v) heat-inactivated calf serum and 100 units/ml penicillin G and 100 μg/ml streptomycin. Cells were grown in 12-well plates to confluence in 3 to 4 days. DNA was transiently transfected by the calcium phosphate-DNA coprecipitation method. The ratio of plasmid used was 2.5 μg of CYP7A1/luciferase reporter gene, 0.5 μg of pCMVβ as internal standard for transfection efficiency, and 0.5 μg of receptor

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1 The abbreviations used are: CYP7A1, cholesterol 7α-hydroxylase gene; CYP27A1, sterol 27-hydroxylase; CYP8B1, sterol 12α-hydroxylase; BARE, bile acid response element; LXR, liver orphan receptor; nt, nucleotides; FXR, farnesoid X receptor; DCA, deoxycholic acid; UDCA, ursodeoxycholic acid; CA, cholic acid; CDA, chenodeoxycholic acid; EMSA, electrophoretic mobility shift assay; RXR, retinoid X receptor; EcR, eddyson receptor; CHO, Chinese hamster ovary; CAT, chloramphenicol acetyltransferase; IBABP, ileal bile-acid-binding protein gene.
expression plasmid. Cells were treated with the indicated concentrations of bile acids, the RXR-specific ligand LG100268 (100 nM, Ligand Pharmaceuticals, La Jolla, CA), or farnesol (50 µM). Cells were harvested 40 h after glycerol shock, washed twice with phosphate-buffered saline and lysed with reporter lysis buffer (Promega). Luciferase activities were measured by luminometer (Lumat model LB9501, Berthold System, Inc., Pittsburgh, PA) and normalized by dividing the relative light units by β-galactosidase activity. Statistical analyses were performed using Sigma plot software. Each assay was done in triplicate, and individual experiments were repeated at least three times.

Chinese hamster ovary (CHO) K1 cells were grown in Dulbecco’s modified Eagle’s medium/nutrient mixture F-12 (1:1) with 5% fetal bovine serum, 5000 units/ml penicillin, and 5000 µg/ml streptomycin (Life Technologies) in a water-jacketed incubator held at 37 °C with a 5% CO₂ atmosphere. An FXR-dependent transactivation assay was assembled in CHO K1 cells similar to that described in Forman et al. (9). Cells were seeded in 6-well culture plates (Falcon) at 1.5·10⁴ cells/well and reporters (19). Each well received 12.5 µg of edesine response element fused to the upstream of the mouse mammary tumor virus promoter/CAT reporter gene. As shown in Fig. 1, DCA (3α, 12α) and CDCA (3α, 7α) at 25 µM activated FXR by 13- and 17-fold, respectively. Glyco- and tauro- conjugates of DCA and CDCA were inactive, as were cholic acid (CA; 3α, 7α, 12α) and its taurine and glycine conjugates (TCA and GCA), tauroxcholate (TLC), tauroursodeoxycholate (TUDCA, 3α), and its conjugate, tauroursodeoxycholic acid (TUDCA). These data are in agreement with those observed in CV-1 cells that hydrophobic bile acids (CDCA and DCA) are more active FXR ligands than hydrophilic bile acids (12–14). The major hydrophilic bile acids in mice and rats, α- and β-muricholic acids (3α, 6β, 7β), were not able to activate FXR as described by other investigators (12–14). Hydrophobic bile acids are able to penetrate through cell membranes by simple diffusion, whereas sodium taurocholate cotransporter facilitates the transport of tauro conjugates into cells. The absence of a bile acid transporter in CHO cells may explain the inability of conjugated bile acids to induce FXR-dependent transcription.

Effect of Cotransfection of RXRα and RXRα on rat CYP7A1/luciferase Reporter Activity—We reported previously that bile acids repressed CYP7A1/luciferase reporter activity in confluent cultures of HepG2 cells but had no effect on reporter activity in CHO or nonconfluent HepG2 cells (16). Therefore, we studied the effect of FXR on liver-specific CYP7A1 expression in its native cellular context by cotransfecting FXR and RXRα expression plasmids along with a rat CYP7A1/luciferase reporter plasmid into confluent cultures of HepG2 cells. RXRα/FXR heterodimer stimulated the reporter activity of the rat CYP7A1/luciferase plasmid (p-416/Luc) by 2-fold without bile acids (Fig. 2). FXR or RXRα alone also stimulated reporter activity.
activity. Interestingly, the RXRα-selective ligand, LG100268, suppressed the reporter activities stimulated by RXRα/FXR or RXRα alone but not by FXR alone. Farnesol (50 μM), a weak activator of FXR, did not have any effect on the activity stimulated by these receptors. This is in agreement with other reports that farnesol was unable to activate FXR (13, 14). These results suggested that RXRα/FXR was able to stimulate CYP7A1 transcription without the addition of an exogenous FXR ligand. It is possible that the RXR-selective ligand could activate the RXRα homodimer or the RXRα/FXR heterodimer, both of which repress CYP7A1 transcription.

We then studied the effect of CDCA on CYP7A1 transcription in HepG2 cells cotransfected with a rat CYP7A1/luciferase reporter (p-416/Luc) and RXRα/FXR expression plasmids. CDCA (25 μM) repressed rat reporter activity by 50% in HepG2 cells without overexpression of FXR (Fig. 3A). This indicates that either endogenous bile acid receptors were activated or other mechanisms, such as the protein kinase C pathway (20), may be involved in repression of CYP7A1 transcription. When the reporter plasmids were cotransfected with RXRα/FXR, reporter activity was stimulated 100% without bile acids. CDCA repressed the FXR-stimulated activity by 80%. The addition of LG268 (0.1 μM) repressed CYP7A1 reporter activity by 70% (Fig. 3B). The combination of CDCA and LG268 further reduced reporter activity significantly (Fig. 3B). It is clear that a bile acid and/or an RXR-selective ligand can activate RXRα/FXR heterodimer, which acts as a bile acid receptor and represses CYP7A1 transcription. We reported previously that bile acid repression of CYP7A1 transcription was liver-specific because bile acids failed to repress the reporter activity in non-liver cells, Chinese hamster ovary cells (3). Therefore, we carried out the same experiments in CHO cells. The addition of 25 μM CDCA did not affect the reporter activity in confluent CHO cells, in contrast to HepG2 cells (Fig. 3C). However, when CHO cells were transfected with RXRα/FXR, CDCA (25 μM) repressed CYP7A1 promoter activity by 50%. At higher concentrations, CDCA stimulated reporter activity independent of FXR (data not shown). These experiments clearly demonstrated that RXRα/FXR was required for mediating bile acid repression of CYP7A1 promoter activity by physiological concentration of CDCA when non-hepatocytes were used in the transfection assay. Thus endogenous bile acid receptors may be present in HepG2 cells for liver-specific repression of CYP7A1 transcription. The ectopically expressed FXR can function as a bile acid receptor when activated by bile acids in both HepG2 and CHO cells.

Effects of Different Bile Acids on CYP7A1 Promoter Activity—
All bile acid and taurine conjugates tested in our transfected HepG2 cells (25 μM of CDCA, CA, DCA, and UDCA) had some repressive effects on rat CYP7A1 reporter activity (Fig. 4). In general, hydrophobic bile acids (CDCA and DCA) were more effective than hydrophilic bile acids (UDCA and CA) in repression of CYP7A1 reporter activity. Only the inhibitory effects of CDCA and DCA were more pronounced when RXRα and FXR were overexpressed in HepG2 cells. Overexpression of a human liver bile acid transporter in HepG2 cells did not affect the repression of CYP7A1/luciferase reporter activity by these bile acids and FXR (data not shown).

The dose responses of CDCA on the inhibition of rat CYP7A1 promoter activity were studied. The concentration of CDCA that required for inhibition of 50% (IC50) of promoter activity was estimated to be approximately 25 μM (Fig. 5), consistent samples. Statistic significance analyses between LG268 and CDCA versus LG268 (p < 0.002) and versus CDCA alone (p < 0.007) were done with Student’s t test.
with that reported previously (12–14). When RXRα and FXR were cotransfected, the IC₅₀ was estimated to be approximately 10 μM. To test the specificity of FXR in mediating bile acid response, we also did the same experiment with HNF4 and COUP-TFII. These two orphan receptors activated CYP7A1 gene, but overexpression of these two receptors in HepG2 cells did not enhance the CDCA response (data not shown).

Identification of an FXR Response Element in CYP7A1 Gene—We have demonstrated previously that BARE-II located between nt −149 and −118 of the rat CYP7A1 promoter was a major bile acid response element. BARE-I, located between nt −75 and −54 only played a minor role in the bile acid response but could confer the bile acid repression to a heterologous promoter (2, 3). To map the FXR response element, we deleted nt from −74 to −54 in BARE-I or −148 to −128 in BARE-II or both sequences from wild type p-416/Luc plasmid and measured the inhibitory effects of CDCA and FXR on CYP7A1 transcriptional activity. The reporter activity of the wild-type plasmid was stimulated by FXR and repressed by CDCA (Fig. 4). Deletion of nucleotides from −74 to −54 in the rat CYP7A1/luc plasmid (p-416Δ BARE-I) greatly stimulated basal promoter activity as we reported previously (3). Cotransfection with RXRα and FXR did not stimulate basal promoter activity, but the bile acid repression was still observed (Fig. 4). These results suggested that the sequences from nt −74 to −54 conferred the stimulation by FXR in the absence of bile acids and confirmed that the deleted sequence is not important in mediating bile acid response with or without FXR. When a sequence from nt −148 to −128 of BARE-II was deleted (p-416Δ BARE-II), CDCA still inhibited promoter activity. However, CDCA did not repress promoter activity when overexpressed with RXRα/FXR. When both BARE-I and BARE-II were deleted (p-416Δ BARE-I +II), similar results were obtained as with p-416Δ BARE-II. These experiments revealed that the FXR response element was localized in nucleotides −148 to −128 and also suggested that endogenous bile acid receptors in HepG2 cells were able to repress CYP7A1 transcription via sequences other than the region from −148 to −128.

Human and Hamster CYP7A1 Were Also Repressed by CDCA-activated RXRα/FXR—We next studied the effect of RXRα/FXR on human and hamster CYP7A1/luc reporter activity in HepG2 cells. Fig. 5 shows that CDCA strongly repressed the reporter activities of two human constructs, ph-1887/Luc and ph-371/Luc and hamster p-1607/Luc, without cotransfection of RXRα/FXR. In contrast to their effect on rat reporter activity, cotransfection of RXRα/FXR reduced the basal promoter activity of both human and hamster reporter plasmids. Reporter activities of both the human and hamster CYP7A1/luc genes were inhibited 80% by CDCA-activated RXRα/FXR.

RXRα/FXR Interactions with BAREs—We performed EMSA to study interaction of FXR with BARE-I and BARE-II sequences. As a positive control, in vitro synthesized RXRα/FXR heterodimer bound to an inverted repeat of AGGTCA with one-base spacing (IR1 of hsp27 EceRE). RXRα/FXR bound rather weakly to the BARE-I probe (nt −74/−53) (Fig. 8A). FXR or RXRα alone did not bind to these probes. However, the BARE-II probe (nt −149/−118) did not bind to in vitro synthesized RXRα/FXR, despite that BARE-II was shown to be a FXR response element by transfection assays. Rat and rabbit were found to have identical sequences in the BARE-I region, whereas mouse and hamster have identical half-site sequences as the rat and rabbit except several nucleotides located between two half-sites. The human corresponding sequences do not have the DR4 motif because the absence of a G at the 3’ position of the 5’ half-site TGGTCA (Fig. 8B). The mouse and hamster sequences did bind FXR, but the corresponding human sequences did not.

**DISCUSSION**

We have studied the effect of bile acid activated-FXR on CYP7A1 transcriptional regulation in HepG2 cells. Results from this study and others (12–14) provide strong evidence that FXR is activated by bile acids and functions as a bile acid receptor to repress CYP7A1 transcription. These results support our receptor-mediated mechanism for bile acid feedback...
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hamster CYP7A1/luc 118) on the rat CYP7A1/luc reporter activity without (−) or with cotransfection of RXRs/FXR expression plasmids in HepG2 cells. BARE-1 (−74/−54) and BARE-II (−148/−118) sequences were deleted from the wild-type p-416/Luc to generate p-416 (ΔBARE-I/Luc) and p-416 (ΔBARE-II/Luc), respectively. The plasmid p-416 (ΔBARE-I-II/Luc) had both BARE-I and BARE-II deleted. CDCA (25 μM) was added in cell culture for 40 h.

Our results showed that all bile acids and conjugates tested were able to inhibit CYP7A1 transcription in confluent HepG2 cells with or without cotransfection with FXR. In general, hydrophobic bile acids are more effective than hydrophilic bile acids in inhibition of CYP7A1 transcription, consistent with in vitro and in vivo studies (1, 12–14, 23–25). However, conjugated bile acids were not able to active FXR in CV-1 cells. When liver bile acid transporter was cotransfected in CV-1 cells, conjugated bile acids were able to activate FXR (12). It is clear that the absence of conjugated bile acid transporters in non-liver cells explain the discrepancy. We observed that cotransfection of FXR was required for inhibition of CYP7A1 transcription by physiological concentration of CDCA in CHO cells, thus explaining our previous observation that bile acid inhibition was liver-specific. Furthermore, other liver-specific transcription factors are also required for FXR down-regulation of CYP7A1 transcription. CDCA was shown to be effective in both activation of FXR by transfection assays and enhancing of FXR interaction with steroid receptor coactivator-1 by fluorescence resonance energy transfer assay (14).

One possible mechanism for FXR mediated down-regulation of CYP7A1 transcription is that FXR may compete with other orphan receptors for the common partner, RXR, hence suppressing gene transcription without DNA binding (squelching effect). The BARE-II of the rat gene contains a DR5, which binds FXR/retinoid acid receptor-α (5). In contrast, human and hamster CYP7A1 are not stimulated by retinoic acid because they lack a DR5 motif (18). The corresponding sequence in the human gene instead binds CPF (26) and BTEB, a member of Sp1 family of transcription factors (27). Therefore, FXR may interact with CPF or BTEB and prevent them from activating human CYP7A1 transcription. Another possibility is that FXR may compete with other nuclear receptors for limiting coactivators such as steroid receptor coactivator-1. One candidate, HNF4, binds to the DR1 motif in BARE-II and plays a role in the transactivation of CYP7A1 transcription (5).

Interestingly, bile acid-activated FXR also can function as a positive transcription factor to stimulate the transcription of the ileal bile acid-binding protein gene (IBABP) (13). Different mechanisms for the negative regulation of the CYP7A1 observed in the liver and positive regulation of the IBABP in the intestine may exist. It is possible that FXR binding to the IR1 motif in IBABP efficiently recruits coactivator, which stimulates gene transcription. The absence of an IR1 in CYP7A1 may preclude FXR from binding such that its negative regulation occurs by one of indirect mechanisms described above.

We observed that FXR, RXRs, or RXRs/FXR stimulated the rat CYP7A1 transcription in the absence of ligand. This is not the case for human CYP7A1, which lacks the DR4 motif. Many orphan receptors such as LXRs are able to stimulate gene
transcription without ligand binding (28). Upon binding of RXR-selective ligand LG100268 to RXRs or CDCA to FXR, RXRs/FXR heterodimers interact with coactivators (12). Therefore, RXRs/FXR heterodimer is activated by the selective ligands of either receptors. Binding of both ligands further enhances receptor activity. This finding is interesting since LG268 was reported to activate some RXR heterodimers including peroxisome proliferator-activated receptor α/RXRs and RXRs/LXRs (29). We observed that peroxisome proliferator-activated receptor α/RXRs inhibited CYP7A1 reporter activity in HepG2 cells by interfering with HNF4 binding to the gene (30). We suggest that there is a general mechanism for negative regulation of the CYP7A1 by these RXR heterodimers.

It should be emphasized that the promoter context and liver-specific expression are important factors to be considered when dissecting CYP7A1 transcriptional control elements. Endogenous nuclear receptors are expressed at very low levels in HepG2 cells but may mask some of the results obtained by overexpressing FXR. Experiments performed in non-liver cells are often difficult to interpret because they lack liver-specific transcription factors responsible for regulating CYP7A1 transcription. Receptor activation and ligand binding assays are usually performed in non-liver cells (CV-1) using the reporter containing several copies of the cognate response element. These assays are useful for identification of ligands for orphan nuclear receptors, but it is necessary to verify these experiments in a liver cell line with a reporter containing the native CYP7A1 promoter.

It is significant that our results suggest the presence of endogenous bile acid receptors in HepG2 cells. It is possible that a family of bile acid receptors may be present in the liver. The identification of FXR as a bile acid receptor is an important step toward the elucidation of the molecular mechanism of bile acid feedback regulation of bile acid synthesis. It also provides a strategy for screening cholesterol-lowering drugs targeted to genes in bile acid synthesis pathways. FXR antagonists should stimulate the conversion of cholesterol to bile acids by stimulating CYP7A1, CYP27A1, and CYP8B1 transcription and also reducing bile acid reabsorption by inhibiting IBAB gene expression (13, 31).

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FIG. 8. EMSA of RXRs and FXR uses rat BARE-I (~74/–53) and BARE-II (~149/–118) probes. In vitro synthesized FXR and RXRs were incubated with labeled probe as indicated. Electrophoresis was performed as described under “Experimental Procedures.” IR1 of EcRE of hep27 gene was used as a positive control of EMSA with RXRs/FXR. A, EMSA of the rat BARE-I and BARE-II probed with FXR. B, EMSA of the rat, mouse, hamster, and human BARE-I probed with FXR. Alignment of the corresponding nucleotide sequences is shown on the bottom of the figure. Rabbit sequences are the same as rat. FXR, RXR.
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