# Identification of intermediates in the bile acid synthetic pathway as ligands for the farnesoid X receptor

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**Abstract** **Bile acid synthesis from cholesterol is tightly regulated via a feedback mechanism mediated by the farnesoid X receptor (FXR), a nuclear receptor activated by bile acids. Synthesis via the classic pathway is initiated by a series of cholesterol ring modifications and followed by the side chain cleavage. Several intermediates accumulate or are excreted as end products of the pathway in diseases involving defective bile acid biosynthesis. In this study, we investigated the ability of these intermediates to activate human FXR. In a cell-based reporter assay and coactivator recruitment assays in vitro, early intermediates possessing an intact cholesterol side chain were inactive, whereas 26- or 25-hydroxylated bile alcohols and $C_{27}$ bile acids were highly efficacious ligands for FXR at a level comparable to that of the most potent physiological ligand, chenodeoxycholic acid. Treatment of HepG2 cells with these precursors repressed the rate-limiting cholesterol 7α-hydroxylase mRNA level and induced the small heterodimer partner and the bile salt export pump mRNA, indicating the ability to regulate bile acid synthesis and excretion. Because 26-hydroxylated bile alcohols and $C_{27}$ bile acids are known to be evolutionary precursors of bile acids in mammals, our findings suggest that human FXR may have retained affinity to these precursors during evolution.**—Nishimaki-Mogami, T., M. Une, T. Fujino, Y. Sato, N. Tamehiro, Y. Kawahara, K. Shudo, and K. Inoue. **Identification of intermediates in the bile acid synthetic pathway as ligands for the farnesoid X receptor.** ***J. Lipid Res.*** 45: **1538–1545.**

**Supplementary key words** bile alcohol • liver X receptor • cholesterol 7α-hydroxylase • small heterodimer partner • bile acid export pump

Cholesterol is converted into two primary bile acids in the mammalian liver: cholic acid (CA) and chenodeoxycholic acid (CDCA). This conversion represents the major route for elimination of cholesterol from the body and plays the primary role in cholesterol homeostasis. The rate of conversion is under strict regulation (1, 2). Accumulation of bile acids in the liver represses expression of cholesterol 7α-hydroxylase (CYP7A1), the initial and rate-limiting-step enzyme in the main pathway (the classic pathway) of bile acid synthesis (3, 4). Recent studies have demonstrated that this feedback regulation is mediated by the nuclear receptor farnesoid X receptor (FXR) (5–7), which is activated by bile acids and their corresponding conjugates at physiological concentrations (8–10). Activation of FXR induces an orphan nuclear receptor, the small heterodimer partner (SHP), which binds to and inhibits liver receptor homolog-1, thereby repressing transcription of CYP7A1 (6, 7) and sterol 12α-hydroxylase (11), an essential enzyme for CA synthesis. Activation of FXR also enhances elimination of bile acids from the liver by inducing gene expression of the bile acid export pump (BSEP) and multidrug-resistance-associated protein 2 (MRP2), which functions to export bile acids or their conjugates into bile (12, 13). Conversely, expression of CYP7A1 in rodents is stimulated by cholesterol feeding (2). The liver X receptor α (LXRα), a nuclear receptor activated by cholesterol metabolites, directly enhances CYP7A1 transcription (14).

Bile acid synthesis from cholesterol requires numerous enzymes located in several organelles and is accomplished via two pathways. Synthesis via the classic pathway includes

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Abbreviations: BSEP, bile acid export pump; CA, cholic acid; CDCA, chenodeoxycholic acid; CTX, cerebrotendinous xanthomatosis; CYP27, sterol 27-hydroxylase; CYP7A1, cholesterol 7α-hydroxylase; DHC, 5β-cholestane-3α,7α-diol; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; FXR, farnesoid X receptor; FXRE, FXR response element; LBD, ligand binding domain; LXR, liver X receptor; LXRE, LXR response element; MRP2, multidrug resistance-associated protein 2; 26-OH-DHC, 5β-cholestane-3α,7α,26-triol; 24-OH-DHCA, 3α,7α,24-trihydroxy-5β-cholestanoic acid; 25-OH-THC, 5β-cholestane-3α,7α,12α,25-tetrol; 26-OH-THC, 5β-cholestane-3α,7α,12α,26-tetrol; 24-OH-THCA, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid; SHP, small heterodimer partner; SPR, surface plasmon resonance; THC, 5β-cholestane-3α,7α,12α-triol; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; $\Delta^{24}$-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid.

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a series of cholesterol ring modifications and oxidative cleavage of the side chain (**Fig. 1**), whereas synthesis via the alternative (acidic) pathway is initiated by hydroxylation of the side chain by sterol 27-hydroxylase (CYP27) and followed by 7α-hydroxylation by oxysterol 7α-hydroxylase (15). Although 27-hydroxycholesterol, the product of the first step in the acidic pathway, has been identified as a ligand of LXR (16, 17), CDCA, the end product of the bile acid biosynthesis, is the most potent physiological ligand for FXR (8–10). Several intermediates accumulate or are excreted as end products of the pathway in diseases involving defective bile acid biosynthesis. In inherited peroxisomal disorders, such as Zellweger syndrome, the $C_{27}$ bile acid intermediates 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) and 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) are produced instead of normal ($C_{24}$) bile acids (18–20). In cerebrotendinous xanthomatosis (CTX), a bile acid synthesis disorder caused by CYP27 deficiency, early intermediates and cholestanol accumulate in a variety of tissues, and glucuronides of 25-hydroxylated bile alcohols are released in bile, blood, and urine (21). In addition, 5β-cholestane-3α, 7α, 12α, 26-tetrol (26-OH-THC) [**2**] and THCA are known to be end products of cholesterol catabolism in primitive vertebrates and are considered to be evolutionary precursors of CA (22). Furthermore, intracellular levels of 26-OH-THC [**2**] have been shown to be comparable to those of CA and CDCA in cultured human hepatocytes (23). In the present study, we investigated whether these intermediates in the bile acid synthetic pathway possess the ability to activate human FXR and whether they have the ability to contribute to the regulation of bile acid synthesis and clearance.

## EXPERIMENTAL PROCEDURES

### Cholanoids

Cholic acid (CA), chenodeoxycholic acid (CDCA), 5-cholestene-3β,7α-diol (7α-hydroxycholesterol), and 5-cholestene-3β,26-diol (27-hydroxycholesterol) [**11**] were purchased from commercial sources. (25S)-3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA) [**3**], 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid ($\Delta^{24}$-THCA) [**4**], 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (24-OH-THCA) [**5**], (25RS)-3α,7α-dihydroxy-5β-cholestanoic acid (DHCA) [**8**], and 3α,7α,24-trihydroxy-5β-cholestanoic acid (24-OH-DHCA) [**9**] were synthesized as described previously (24, 25). (25R)-5β-cholestane-3α,7α,12α,26-tetrol (26-OH-THC) [**2**] and (25RS)-5β-cholestane-3α,7α,26-triol (26-OH-DHC) [**7**] were prepared by reduction with $LiALH_4$ from the corresponding acids. 5β-cholestane-3α,7α,12α-triol (THC) [**1**] (26), 5β-cholestane-3α,7α-diol (DHC) [**6**] (26), and

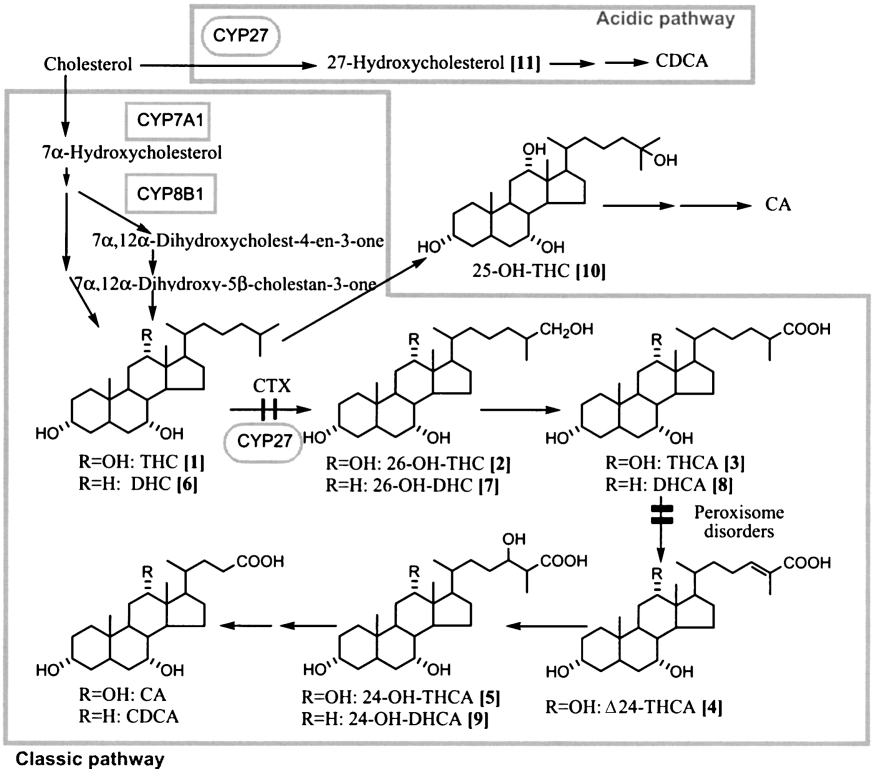


**Fig. 1.** Intermediates of bile acid synthesis. Compounds tested for the ability to activate farnesoid X receptor (FXR) are indicated. **1**, 5β-cholestane-3α,7α,12α-triol (THC); **2**, 5β-cholestane-3α,7α,12α,26-tetrol (26-OH-THC); **3**, 3α,7α,12α-trihydroxy-5β-cholestanoic acid (THCA); **4**, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid ($\Delta^{24}$-THCA); **5**, 3α,7α,12α,24-tetrahydroxy-5β-cholestanoic acid (24-OH-THCA); **6**, 5β-cholestane-3α,7α-diol (DHC); **7**, 5β-cholestane-3α,7α,26-triol (26-OH-DHC); **8**, 3α,7α-dihydroxy-5β-cholestanoic acid (DHCA); **9**, 3α,7α,24-trihydroxy-5β-cholestanoic acid (24-OH-DHCA); **10**, 5β-cholestane-3α,7α,12α,25-tetrol (25-OH-THC); **11**, 5-cholestene-3β,26-diol (27-hydroxycholesterol). CA, cholic acid; CDCA, chenodeoxycholic acid. Compounds in the figure are indicated by bracketed numbers.

5β-cholestane-3α,7α,12α,25-tetrol (25-OH-THC) [**10**] (27) were synthesized as described previously.

### Plasmid constructs

Plasmids for an FXR response element (FXRE)-driven luciferase reporter (pFXRE-tk-Luc) and an LXR response element (LXRE)-driven luciferase reporter were constructed by inserting the cDNAs containing four copies of FXRE from phospholipid transfer protein promoter (28) or two copies of LXREa and LXREb from the sterol response element binding protein-1c promoter (29), respectively, upstream from the thymidine kinase (tk) promoter. cDNAs encoding full-length human FXR, RXRα, LXRα, and LXRβ, were PCR cloned and inserted into mammalian expression vector pcDNA3.1 (Invitrogen).

### Transient transfections and reporter gene assays

CV-1 cells were maintained in DMEM containing 10% FCS and 100 μg/ml kanamycin and seeded in 24-well plates 24 h prior to transfection. Cells were transfected with 187.5 ng of pFXRE-tk-Luc, 62.5 ng each of pcDNA3.1-FXR and pcDNA3.1-RXRα, and 187.5 ng of pSV-β-galactosidase control vector (Promega) with PolyFect (Qiagen). Three hours after transfection, cells were exposed to bile acids or bile acid precursors at concentrations of 0 to 100 μM in the medium containing 10% FCS for 24 h. For the assay of LXR activation, CV-1 cells were transfected with 248 ng of pLXRE-tk-Luc, 1.25 ng each of pcDNA3.1-LXR and pcDNA3.1-RXRα, and 248 ng of pSV-β-galactosidase control vector with PolyFect. Three hours after transfection, cells were incubated in the medium containing 10% delipidated FBS, 50 μM compactin, 10 μM mevalonic acid, and various concentrations of bile acid precursors for 24 h. Cells were lysed, and luciferase activity was determined with Steady-Glo reagent. Luciferase activity was normalized to β-galactosidase activity for each well.

### Coactivator association assay using surface plasmon resonance

The analyses were performed using BIAcore 3000 opitical biosensors (BIAcore AB, Uppsala, Sweden) as described previously (30). Briefly, biotinylated wild-type peptide from human SRC-1 (CPSSHSSLTARHKILHRLLQEGSPS-$CONH_2$) containing the LXXLL nuclear receptor interaction motif and consensus-mutated peptide (CPSSHSSLTARHKIAHRALQEGSPS-$CONH_2$) were immobilized on the surfaces of streptoavidin chips (BIAcore AB). Human FXR LBD (LBD) (1–4 μM), which was expressed in *Escherichia coli* as a glutathione S-transferase (GST) fusion protein and purified on glutathione beads after cleaving with precision protease, was preincubated with 100 μM ligands for 1 h and injected over the surfaces in a running buffer composed of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 0.05% Tween 20, and 0.5% DMSO at 25°C. After completion of the injection (120 s), the complex formed was washed with buffer for an additional 120 s. The chip surfaces were regenerated down to the peptide level by subsequent application of a 30 s pulse of 0.1% SDS and 10 mM NaOH. To eliminate responses attributable to nonspecific interactions, sensorgrams detected with a wild-type SRC-1-immobilized chip were routinely corrected using sensorgrams obtained with a chip immobilized with mutant SRC1. Kinetic parameters were determined as described previously (30). Briefly, the apparent association rate constant ($K$) was determined by nonlinear regression analysis of the initial part of the association phase with PRISM software (GraphPad Software, Inc.). The $K$ values obtained from sensorgrams for four different concentrations of FXR LBD were plotted against the concentration ($C$), and the rate constants for association ($k_a$) and dissociation ($k_d$) were obtained from the equation $K = k_aC + k_d$.

### Coactivator association assay using fluorescence polarization

The assay was performed essentially according to the published procedure (31). TAMRA-labeled peptide (100 nM, with amino acid sequence ILRKLLQE) was incubated for 1 h with purified GST-fused human FXR LBD or human LXR LBD (1.5 μM) and candidate ligands in 100 μl of buffer (10 mM Hepes, 150 mM NaCl, 2 mM $MgCl_2$, and 5 mM DTT at pH 7.9) in a black polypropylene 96-well plate on a shaker. Ligand-dependent recruitment of the coactivator peptide was measured as increases in fluorescence polarization with a Fusionα-FP (Perkin Elmer Life Science).

### HepG2 cell culture and real-time quantitative RT-PCRs

HepG2 cells were maintained in DMEM containing 10% FCS and treated with bile acids or bile acid precursors in DMEM containing 0.5% delipidated FBS for 20 h. Cells were harvested, and total RNA was extracted using the RNeasy Mini Kit (Qiagen). The RNA samples were treated with DNAase according to the manufacturer's protocol (Qiagen). Relative expression levels of mRNA were determined using the TaqMan one-step RT-PCR Master Mix Reagent Kit and the ABI Prism 7700 sequence detection system (Applied Biosystems). Primer/probe sequences used were as follows: human SHP forward primer, 5′-GGTGCAGTGGCTTCAATGC-3′; reverse primer, 5′-GGTTGAAGAGGATGGTCCCTTT-3′; probe, 5′FAM- TCTGGAGCCTGGAGCTTAGCCCCA-TAMRA3′. The primer/probe sequences for human BSEP and human CYP7A1 were the same as described previously (32). Expression data were normalized to GAPDH mRNA levels, and presented as the fold difference of treated cells against untreated cells.

## RESULTS

### Activation of FXR by intermediates of bile acid biosynthetic pathways

We assessed the ability of a series of $C_{27}$ intermediates in bile acid synthesis (Fig. 1) to activate human FXR using a transient transfection assay with a luciferase reporter plasmid containing a synthetic FXR response element, along with expression plasmids for FXR and RXRα. In the classic pathway for CA biosynthesis, early intermediates in the cholesterol ring modification step, such as 7α-hydroxycholesterol, 7α,12α-dihydroxycholest-4-ene-3-one, and THC [**1**], were inactive (**Fig. 2A**). In contrast, 26-OH-THC [**2**], the subsequent side chain hydroxylation product by CYP27 (33), exhibited higher activity than that of the most potent physiological ligand, CDCA. Similarly, although DHC [**6**], a precursor of CDCA possessing a CDCA type of nucleus but an intact cholesterol side chain, was inactive, 26-OH-DHC [**7**], its hydroxylation product by CYP27, and DHCA [**8**], the further oxidation product having a $C_3$-unit-longer side chain than CDCA, exhibited activity comparable to that of CDCA. In contrast, 27-hydroxycholesterol [**11**], which is also produced by CYP27 in the acidic pathway (15), was inactive. Notably, although CA was inactive, as previously reported (8–10), THCA [**3**] and $\Delta^{24}$-THCA [**4**], the immediate precursors of CA, exhibited substantial activity. Alternatively, THC [**1**] can be hydroxylated by CYP3A (34), and the formed 25-OH-THC [**10**] is eventually converted to CA (Fig. 1). As shown in Fig. 2A, the 25-OH-THC [**10**] exhib-

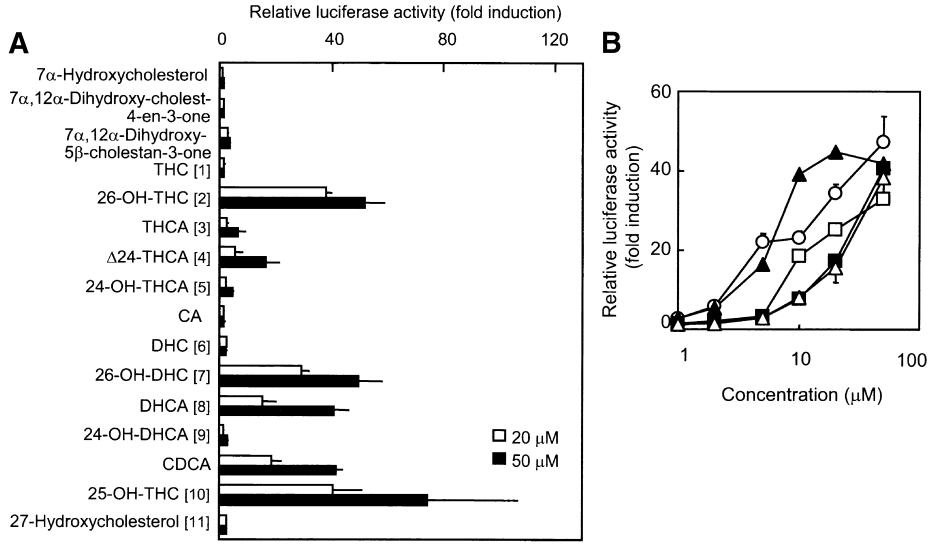


**Fig. 2.** Activation of FXR by intermediates in bile acid synthetic pathways. A: CV-1 cells were transfected with expression plasmids for human FXR and RXRα and the $FXRE_{PLTP}$x4-tk-luc reporter plasmid, together with a β-gal internal control. Cells were treated with vehicle alone or 20–50 μM of the indicated bile acid precursors, as indicated. Luciferase activity in the cell extract was normalized using the β-gal internal control and expressed as fold induction relative to vehicle-treated cells. Data represent the means ± SD of three independent experiments, which were done in triplicate. B: Dose-response of bile acid precursor for activation of a reporter gene by FXR. Cells were transfected as in (A) in the presence of increasing concentrations of 26-OH-THC [**2**] (open circles), 25-OH-THC [**10**] (closed triangles), 26-OH-DHC [**7**] (open boxes), DHCA [**9**] (open triangles), and CDCA (closed boxes). Data represent the mean ± SD of three data points. Compounds in the figure are indicated by bracketed numbers.

ited strong activity. This pathway is the only route of bile acid synthesis in CTX caused by CYP27 deficiency (35).

Dose-response analysis showed that the $C_{27}$ bile alcohols, 26-OH-THC [**2**], 26-OH-DHC [**7**], and 25-OH-THC [**10**], elicited an effect equivalent to that of CDCA at lower concentrations (Fig. 2B). The dose-response relation of DHCA was similar to that of CDCA.

Although 27-hydroxycholesterol [**11**], a known LXR ligand (16, 17), led to ~6-fold activation of LXRα or LXRβ at 20 μM in transient transfection assays, 26-OH-THC [**2**] and 26-OH-DHC [**7**], CYP27 metabolites, exhibited no activity (**Fig. 3**), and the FXR-activating intermediates were all inactive as well.

### Bile acid precursors are FXR ligands

We performed in vitro coactivator recruitment assays to determine whether these bile acid precursors bind directly to FXR. In an assay using surface plasmon resonance (SPR), ligand-induced interaction of FXR LBD with an LXXLL peptide from coactivator SRC-1 was detected as a change in the refractive index (**Fig. 4**). CDCA exhibited the most potent interaction (Fig. 4B). The CDCA precursors, 26-OH-DHC [**7**] and DHCA [**8**] (Fig. 4B), and the CA precursors, THCA [**3**] and $\Delta^{24}$-THCA [**4**] (Fig. 4A), also caused strong interactions. Kinetic analysis revealed that these precursors increased the affinity of FXR for an SRC-1 peptide (as shown by decreased $K_d$ values) at levels comparable to that of CDCA (**Table 1**). 25-OH-THC [**10**], a characteristic metabolite that accumulates in CTX, produced a moderate response (Fig. 4C). DHC [**6**] (Fig. 4B), THC [**1**], and CA (Fig. 4A) were inactive. These findings are consistent with the results obtained from the cell-based luciferase assay (Fig. 2A), except for a strong response evoked by 24-OH-DHCA [**9**] (Fig. 4B) and a very weak response by 26-OH-THC [**2**] (Fig. 4A). In another coactivator association assay that detects interactions between FXR LBD and a fluorescence-labeled peptide as a change in fluorescence polarization, 26-OH-THC [**2**] caused partial activation with half-maximum activation at ~10 μM (Fig. 4D). CA also caused a

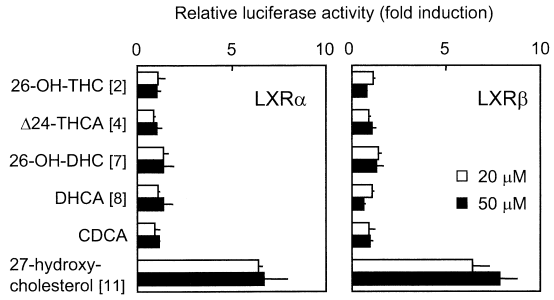


**Fig. 3.** FXR-activating bile acid precursors do not activate LXRα or LXRβ. CV-1 cells were transfected with expression plasmids for human LXRα (or LXRβ), RXRα, and the $LXRE_{SREBP\text{-}1c}$x4-tk-luc reporter and treated with bile acid precursors as in Fig. 2A. Luciferase activity was normalized using the β-gal internal control and expressed as fold induction relative to vehicle-treated cells. The data are shown as the means ± SD of six data points. Compounds in the figure are indicated by bracketed numbers.

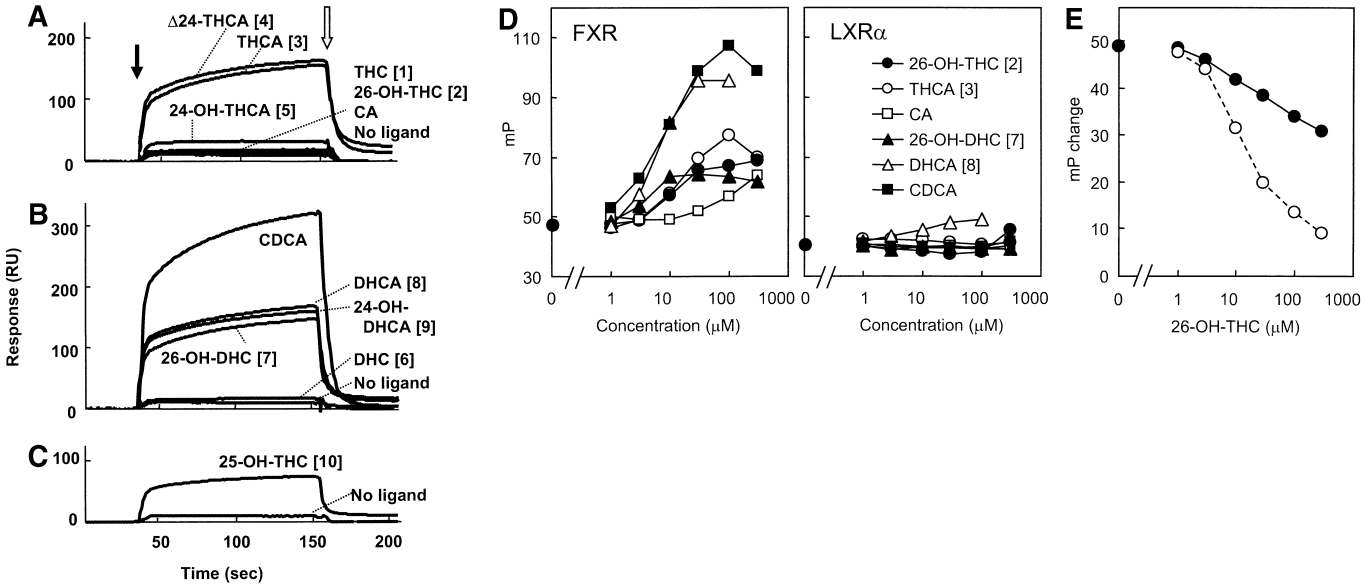


**Fig. 4.** Bile acid precursors promote association of FXR with SRC-1 peptide in vitro as determined by surface plasmon resonance (SPR) (A–C) or fluorescence polarization assay (D and E). A–C: for SPR assay, FXR LBD (4 μM) preincubated for 1 h with vehicle (DMSO) alone or 100 μM CDCA, CA, or intermediates of bile acid synthetic pathways was injected over the sensorchip surface immobilized with SRC-1 peptide. Ligand-induced association of the FXR LBD with SRC-1 peptide was monitored by a change in resonance units (RUs). The sensorgrams shown have been corrected for use of a surface immobilized with a mutant peptide. The arrow and the double arrow indicate the beginning and end, respectively, of the injections. Sensorgrams shown for CA and its precursors (A), CDCA and its precursors (B), and cerebrotendinous xanthomatosis-associated 25-OH-THC [**10**] (C) were from a typical series of three experiments performed. D: For fluorescence polarization assay, a fluorescence-tagged SRC-1 peptide (0.1 μM) was incubated with 1.5 μM of GST-FXR or GST-LXRα in the presence of various concentrations of CDCA, CA, or their precursors. Ligand-induced SRC-1 peptide association with the receptor was monitored by increases in millipolarization fluorescence units (mP). E: 26-OH-THC [**2**] competes with CDCA for FXR binding as measured by fluorescence polarization assay. Changes in fluorescence polarization caused by 50 μM CDCA in the presence of the concentrations of 26-OH-THC [**2**] indicated are shown (closed circles). A blank value in the absence of ligands was subtracted from measured values. A value for fluorescence polarization caused by 26-OH-THC [**2**] in the absence of CDCA was subtracted from the measured value at each concentration (open circles). Compounds in the figure are indicated by bracketed numbers.

small response at 100–300 μM, whereas DHCA [**8**] caused an association as strong as CDCA. 26-OH-DHC [**7**] led to a substantial association that peaked at 10 μM. In contrast, no interaction between LXR LBD and the peptide was evoked by these compounds (Fig. 4D). When 26-OH-THC [**2**] was assayed in the presence of 50 μM CDCA, the change in fluorescence polarization elicited by CDCA (Fig. 4E) was reduced by 40% by this compound, indicating displacement of CDCA from FXR. The reduction reached 80% when the change in fluorescence polarization elicited by 26-OH-THC [**2**] in the absence of CDCA was subtracted from the measured value. Thus, the data show that 26-OH-THC [**2**] binds to FXR strongly but promotes association with SRC-1 poorly.

### Bile acid precursors regulate FXR target genes

We investigated whether the above precursors modulate the expression of endogenous FXR target genes. HepG2 cells were treated with 50 μM of bile acids or their precursors, and mRNA levels were monitored by real-time quantitative RT-PCR. The concentration of precursors was reduced to 20 μM or 30 μM when cytotoxicity, judged on the basis of >30% decrease in GAPDH mRNA or cell proteins, was observed. Although CA had no effect on SHP mRNA expression, 26-OH-THC [**2**] (20 μM) and THCA [**3**] (50 μM) increased the SHP level by 2.4- and 3-fold, respectively (**Fig. 5**). CDCA (50 μM) and the synthetic FXR agonist GW4064 (1 μM) induced 6.4-fold and 3.9-fold, respectively, increases in SHP mRNA expression, and 26-OH-DHC [**7**] (20 μM) and DHCA [**8**] (30 μM) caused 4- and 6.5-fold inductions, respectively. 25-OH-THC (20 μM) led to a 4-fold induction. Expression of BSEP mRNA was markedly induced by these precursors and CDCA, in parallel with the SHP mRNA level. 25-OH-THC [**10**] induced a 3.5-fold increase in BSEP mRNA expression, but the increase was much smaller than expected based on the elevation of the SHP level.

Increased SHP is known to suppress CYP7A1 expression (6, 7). The precursors, CDCA, and GW4064 all caused marked reductions in the CYP7A1 mRNA level, and there was an inverse correlation between the SHP and CYP7A mRNA levels.

## DISCUSSION

Conversion of cholesterol to bile acids is achieved by numerous processes that yield a series of intermediates. In the present study, the results of a cell-based luciferase assay showed that late intermediates in the classic pathway, including 26-OH-THC [**2**], 26-OH-DHC [**7**], DHCA [**8**], and 25-OH-THC [**10**], possess the ability to activate hu-

TABLE 1. Affinity and rate constants for FXR/SRC-1 interactions induced by bile acids and bile acid precursors

| | $k_a$ ($M^{-1}$ $S^{-1}$) | $k_d$ ($S^{-1}$) | $K_d$ (μM) |
|---|---|---|---|
| No ligand | $0.56 \times 10^4$ | $1.97 \times 10^{-1}$ | 35.2 |
| THC [**1**] | $1.01 \times 10^4$ | $2.65 \times 10^{-1}$ | 26.2 |
| 26-OH-THC [**2**] | $1.05 \times 10^4$ | $3.34 \times 10^{-1}$ | 31.8 |
| THCA [**3**] | $3.53 \times 10^4$ | $2.04 \times 10^{-1}$ | 5.78 |
| $\Delta^{24}$-THCA[**4**] | $3.65 \times 10^4$ | $2.03 \times 10^{-1}$ | 5.56 |
| 24-OH-THCA [**5**] | $1.03 \times 10^4$ | $2.16 \times 10^{-1}$ | 21.0 |
| CA | $1.13 \times 10^4$ | $3.35 \times 10^{-1}$ | 29.7 |
| DHC [**6**] | $0.84 \times 10^4$ | $2.22 \times 10^{-1}$ | 26.4 |
| 26-OH-DHC [**7**] | $2.46 \times 10^4$ | $2.04 \times 10^{-1}$ | 8.29 |
| DHCA [**8**] | $3.66 \times 10^4$ | $2.42 \times 10^{-1}$ | 6.61 |
| 24-OH-DHCA [**9**] | $3.36 \times 10^4$ | $2.20 \times 10^{-1}$ | 6.55 |
| CDCA | $4.84 \times 10^4$ | $1.73 \times 10^{-1}$ | 3.57 |
| 25-OH-THC [**10**] | $2.91 \times 10^4$ | $2.43 \times 10^{-1}$ | 8.35 |

CA, cholic acid; CDCA, chenodeoxycholic acid; DHC, 5β-cholestane-3α,7α-diol; DHCA, 3α,7α-dihydroxy-5β-cholestanoic acid; FXR, farnesoid X receptor; THC, 5β-cholestane-3α,7α,12α-triol; 26-OH-THC, 5β-cholestane-3α,7α,12α,26-tetrol; THCA, 3α,7α,12α-trihydroxy-5β-cholestanoic acid; $\Delta^{24}$-THCA, 3α,7α,12α-trihydroxy-5β-cholest-24-enoic acid; 24-OH-THCA, 3α,7α,12α, 24-tetrahydroxy-5β-cholestanoic acid; 26-OH-THC, 5β-cholestane-3α,7α,12α,26-tetrol; 24-OH-DHCA, 3α,7α,24-trihydroxy-5β-cholestanoic acid; 25-OH-THC, 5β-cholestane-3α,7α,12α,25-tetrol. The interactions were monitored using surface plasmon resonance as described in the legend for Fig. 4. Kinetic parameters were determined by analyzing sensorgrams for four different concentrations of FXR LBD (1–4 μM) preincubated with 100 μM bile acids or their precursors.

man FXR at levels comparable to that of the most potent physiological ligand, CDCA (Fig. 2A). Furthermore, their ability to induce coactivator association in vitro (Fig. 4) clearly demonstrated that these intermediates directly activate FXR as ligands without being metabolized to CA or CDCA. 26-OH-THC [**2**] showed no (Fig. 4A) or low (Fig. 4D) SRC-1 peptide-recruiting activity, but potently transactivated FXR in a cellular assay (Fig. 2A). However, its ability to displace CDCA from FXR (Fig. 4E) clearly indicates that this intermediate binds to FXR. THCA [**3**], $\Delta^{24}$-THCA [**4**], and, in particular, 24-OH-DHCA [**9**] were potent agonists of coactivator association in vitro (Fig. 4A, B), and their responses were stronger than expected from the results of the cell-based luciferase assay, suggesting that transportation of these polar acidic intermediates into CV-1 cells is limited. No activity of CA in the luciferase assay is consistent with previous studies (9, 10), and this is thought to be attributable to its limited transportation, because CA requires the coexpression of bile acid transporters for transactivation of the FXR reporter gene in CV-1 cells (9, 10). In addition, a recent study has shown that the binding affinity of CA to human FXR is very low ($IC_{50}$, 586 μM) (36). Taken together, our findings demonstrate that 26- and 25-hydroxyl bile alcohols and $C_{27}$ bile acids, intermediates produced downstream of the cholesterol side chain hydroxylation steps, are potent ligands for FXR.

We showed that the CA precursors 26-OH-THC [**2**], THCA [**3**], and $\Delta^{24}$-THCA [**4**] are far more efficacious agonists of FXR than CA in vitro (Fig. 4). The concentration of 26-OH-THC [**2**] required to induce coactivator association was 10 times lower than that of CA and comparable to that of CDCA (Fig. 4D). A study using cultured primary human hepatocytes (23) has shown that the intracellular level of 26-OH-THC [**2**] is twice as high as that of CA and half that of CDCA, although a 300-fold higher amount of CA than of 26-OH-THC [**2**] is released into the medium. 26-OH-THC [**2**] strongly induced SHP and BSEP mRNA expression and repressed the CYP7A1 mRNA level (Fig. 5). Thus, it is likely that 26-OH-THC [**2**] and end-product $C_{24}$ bile acids regulate their own synthesis and excretion by acting as FXR ligands under certain conditions. The cultured cells may resemble the liver when its supply of bile acids from the intestine via the enterohepatic circulation is disrupted.

Intacellular levels of THCA [**3**] and DHCA [**8**] in normal human hepatocytes have been shown to be 5 to 10 times lower than those of CA and CDCA (23). However, much higher levels of these $C_{27}$ bile acids are present in the plasma of patients with inherited peroxisome disorders, such as Zellweger syndrome, than the levels of CA and CDCA in normal subjects (37, 38), and the concentration of 25-OH-THC [**10**] in the hepatic microsomes of CTX patients is 20- to 100-fold higher than in control subjects (39). Our findings indicate that these precursors modulate FXR target gene expression more potently or

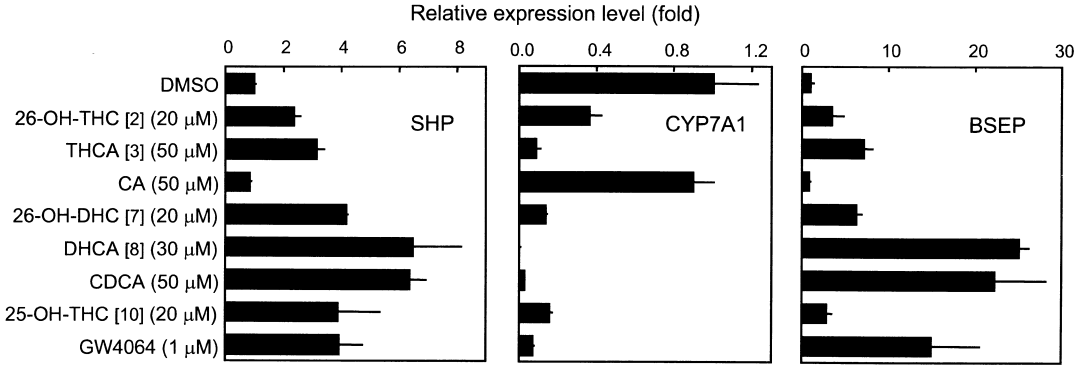


**Fig. 5.** Regulation of gene expression by various bile acid precursors. HepG2 cells were treated for 20 h with vehicle (DMSO) alone, 1 μM GW4064, or the concentrations (20–50 μM) of CDCA, CA, or bile acid precursors indicated. Total RNA was isolated from the cells, and the levels of SHP, CYP7A1, and BSEP mRNA were quantitated by real-time RT-PCR. Data were normalized to GAPDH mRNA levels and are expressed as fold induction relative to vehicle-treated cells. The values represent the means ± SD of three incubations. Compounds in the figure are indicated by bracketed numbers.

efficiently than CA or CDCA (Fig. 5), and thus, these intermediates are likely to regulate their own synthesis and excretion in such patients. Inasmuch as studies have shown that the negative regulation of CYP7A1 levels is achieved by redundant pathways, including repression through activation of the xenobiotic receptor pregnane X receptor or activation of c-Jun N-terminal kinase (2, 40–42), CYP7A1 repression by these intermediates may involve FXR-independent mechanisms. 25-OH-THC [**10**] has been shown to activate mouse PXR but to be ineffective in activating human PXR (43).

Although we showed that 25-OH-THC [**10**] repressed CYP7A1 expression in HepG2 cells, studies have shown that CYP27 deficiency is associated with enhanced CYP7A1 levels (44–46). Studies in vivo using cholestyramine have shown that decreased amounts of bile acids returning to the liver from the intestine induce CYP7A1 expression (1, 2), although the production of bile acids, FXR agonists, would be enhanced in this situation. Bile alcohols, including 25-OH-THC [**10**], are secreted into the bile and urine following glucuronidation (47, 48), and do not undergo enterohepatic circulation (49). Thus, the enhanced CYP7A1 expression in CYP27 deficiency may be the result of decreased flux of bile acids and bile alcohols into the liver, while production of FXR-activating 25-OH-THC [**10**] is increased. The evolution of bile alcohols to bile acids is likely to have provided for regulation of CYP7A1 expression through the enterohepatic circulation.

The coactivator association induced by 26-OH-THC [**2**] or CA was detected more sensitively by the fluorescence polarization assay (Fig. 4D) than by the SPR assay (Fig. 4A). Because SRC-1-derived peptide was immobilized for the SPR assay, conformational or steric restrictions of the SRC-1 peptide may have diminished the association.

Previous studies have shown that conjugation of CDCAs with glycine or taurine only modestly affects their FXR binding affinity and activation efficacy (8–10). A crystal structure study predicted that the carbonyl oxygen at the C-24 position in both conjugated and unconjugated CDCA forms the hydrogen bond with the guanidino group of Arg328 in FXR LBD (50). We have shown that a $C_{24}$ bile alcohol derived by chemical reduction of the carboxyl group of CDCA is a potent ligand for FXR (51). Indeed, this compound possesses the alcoholic oxygen at C-24 that can form a hydrogen bond with Arg328. However, in the present study, we showed that 26-OH-DHC [**7**] and DHCA [**8**], which possess the same nuclear structure as CDCA but lack the C-24 oxygen, exhibit strong ability to activate FXR. Inasmuch as DHC [**6**] was inactive, these findings indicate that the C-26 oxygen plays a critical role in FXR activation. 26-OH-THC [**2**] and 25-OH-THC [**10**] were also highly active, whereas THC [**1**] and 27-hydroxycholesterol [**11**] were inactive, indicating the importance of both a bile-acid type nucleus and an oxygen at the C-25 or C-26 position.

26-OH-THC [**2**] and THCA [**3**] are known to be end products of cholesterol catabolism in several evolutionarily primitive vertebrates (22). The mechanism of conversion of cholesterol to bile acids in mammals is likely to be a recapitulation of the evolution of cholanoids and thus entails the intermediary formation of bile alcohols and $C_{27}$ bile acids. Human FXR may have retained affinity for these precursors during evolution. A recent study has shown that FXR-like orphan receptors (FORs), identified in *Xenopus laevis* liver and kidney, are not activated by bile acids but are activated by bullfrog gallbladder bile extracts (52), which contain bile alcohols (53).

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