

Cholesterol and Bile Acids Regulate Xenosensor Signaling in Drug-mediated Induction of Cytochromes P450*

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Cytochromes P450 (CYP) constitute the major enzymatic system for metabolism of xenobiotics. Here we demonstrate that transcriptional activation of CYPs by the drug-sensing nuclear receptors pregnane X receptor, constitutive androstane receptor, and the chicken xenobiotic receptor (CXR) can be modulated by endogenous cholesterol and bile acids. Bile acids induce the chicken drug-activated CYP2H1 via CXR, whereas the hydroxylated metabolites of bile acids and oxysterols inhibit drug induction. The cholesterol-sensing liver X receptor competes with CXR, pregnane X receptor, or constitutive androstane receptor for regulation of drug-responsive enhancers from chicken CYP2H1, human CYP3A4, or human CYP2B6, respectively. Thus, not only cholesterol 7 α -hydroxylase (CYP7A1), but also drug-inducible CYPs, are diametrically affected by these receptors. Our findings reveal new insights into the increasingly complex network of nuclear receptors regulating lipid homeostasis and drug metabolism.

Cytochromes P450 (CYPs)¹ are heme-containing enzymes responsible for the hydroxylation of lipophilic substrates in all species. In the liver, a subset of members of the CYP gene superfamily metabolize xenobiotics such as drugs, food additives, and pollutants (1). Some of these CYPs can be transcriptionally regulated by their own substrates and by other compounds. The barbiturate phenobarbital (PB) represents a class of inducers that activate predominantly the CYP2B and

CYP2C subfamilies, whereas the glucocorticoid dexamethasone and the antibiotic rifampicin exemplify drugs that elevate CYP3A levels in man. Induction of drug metabolism has important clinical consequences, causing altered pharmacokinetics of drugs and carcinogens, drug-drug interactions, and changes in the metabolism of steroids, vitamin D, and other endogenous compounds. Other types of hepatic CYPs occupy key positions in the biosynthesis and metabolism of numerous endogenous molecules including steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, or retinoids. As examples, CYP51 converts lanosterol into cholesterol, whereas CYP7A1 catalyzes the first step of cholesterol metabolism into bile acids (2). Like xenobiotic-metabolizing CYPs, some of the CYPs that hydroxylate endogenous substrates are also regulated transcriptionally by their substrates or metabolites. In the mouse, CYP7A1 is induced by oxysterols and inhibited by bile acids (3).

Transcriptional regulation of many CYPs is carried out by members of the gene superfamily of nuclear receptors (3, 4). The relative lipophilicity and small size of inducer compounds allows either direct diffusion or facilitated transport into the cell and interaction with specific intracellular receptors, which then bind to their respective DNA recognition elements arranged as repeats of hexamer half-sites in the 5'-flanking regions of CYPs (5, 6). The nuclear receptors liver X receptor (LXR) and the farnesoid X receptor (FXR) bind oxysterols and bile acids, respectively, and are key players in the regulation of CYP7A1 (3). The transcriptional activation of CYP7A1 by LXR in rodents is counteracted by high bile acid levels that activate FXR. FXR subsequently increases the transcription of the small heterodimerization partner that acts as an inhibitor of several nuclear receptors, including LXR (7, 8).

Although induction of CYPs by PB has been described over 40 years ago, our understanding of the molecular mechanism is still fragmentary. Recently, the nuclear receptors constitutive androstane receptor (CAR), pregnane X receptor (PXR) (alternatively called steroid and xenobiotic receptor or pregnane-activated receptor), and chicken xenobiotic receptor (CXR) were discovered to be involved in drug induction of CYP2Bs, CYP3As, and CYP2H1 in humans, mice, and chickens, respectively (4–6, 9). In mammals, CAR and PXR exhibit overlapping substrate and DNA recognition specificity, and the exact contribution of these two receptors to drug induction has not been fully elucidated (10–13). In chickens, only one xenobiotic-sensing orphan nuclear receptor has been identified. It might constitute the ancestral gene that diverged into CAR and PXR in mammals (9). Despite this apparent difference, the molecular mechanism of drug-mediated CYP induction is conserved at the level of both nuclear receptors and DNA recognition elements from birds to humans (9, 14–16).

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¹ The abbreviations used are: CYP, cytochrome P-450; PB, phenobarbital; LXR, liver X receptor; FXR, farnesoid X receptor; CAR, constitutive androstane receptor; PXR, pregnane X receptor; CXR, chicken xenobiotic receptor; RXR, 9-*cis*-retinoic acid receptor; LBD, ligand-binding domain; PBREM, PB-responsive enhancer module; CAT, chloramphenicol acetyltransferase; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; PBRU, PB-responsive enhancer unit; CA, cholic acid; DCA, deoxycholic acid; CCA, chenodeoxycholic acid; HA, hemagglutinin; GGPP, geranylgeranyl-pyrophosphate; UAS, upstream activating sequence; HC, hyocholic acid; HD, hyodeoxycholic acid.

Apparently, many CYPs are responsible for maintaining both lipid homeostasis and detoxification of lipid-soluble drugs and xenobiotics (4). Accordingly, the xenosensor PXR is also activated by endogenous bile acids and involved in hepatic detoxification of excess bile acid levels (13, 17). In this report, we describe experiments concerning the role of xenobiotic-sensing nuclear receptors in lipid homeostasis as well as the role of cholesterol- and bile acid-sensing nuclear receptors in drug metabolism. Moreover, we present a hypothesis on how these nuclear receptors might interact with each other and thus provide a sensitive regulatory network that controls both lipid and xenobiotic levels. These findings also provide insight into the evolution of these systems and suggest that our body might recognize lipophilic xenobiotics as a kind of "toxic bile acids."

EXPERIMENTAL PROCEDURES

Plasmids—Full-length receptor coding sequences from chicken CXR, 9-*cis*-retinoic acid receptor γ (RXR γ), FXR, and LXR were amplified and subcloned into the expression vector pSG5 (Stratagene, Basel, Switzerland). Chicken CXR (amino acids 97–391), FXR (amino acids 194–473), and LXR (amino acids 126–409) ligand binding domains (LBD) fused to the yeast GAL4 transcription factor DBD were obtained by PCR amplification of the LBDs of the nuclear receptors and subsequent subcloning of the PCR products in frame into the expression plasmid pA4.7, a kind gift from Dr. A. Kralli (Division of Biochemistry, Biozentrum, Basel, Switzerland). An N-terminal hemagglutinin (HA) tag was produced using the oligonucleotides 5'-AAT TCC CAT GTA CCC ATA CGA TGT TCC AGA TTA CGC TG-3' and 5'-AAT TCA GCG TAA TCT GGA ACA TCG TAT GGG TAC ATG GG-3' synthesized by Microsynth (Balgach, Switzerland). The double-stranded oligonucleotide was ligated into the *Eco*RI site of the pSG5-CXR expression vector. The (UAS)5-tk-CAT reporter plasmid was generously provided by Dr. S. A. Klierer (Department of Molecular Biology, University of Texas Southwestern, Dallas, TX). Oligonucleotides for the wild type CYP2B6 51-bp PB-responsive enhancer module (PBREM) and the corresponding 51 bp where the hexamer half-sites of the two DR-4 elements were mutated into *Sac*II and *Eco*RV sites, respectively, were synthesized by Microsynth. Similarly, a wild type ER-6 element from the CYP3A4 promoter and a corresponding element with mutations in the intrinsic DR-4 element were obtained. Human LXR α in the CMX expression vector was a kind gift of Dr. R. M. Evans, The Salk Institute, San Diego, CA. Human RXR α expression plasmid was generously provided by Dr. P. Chambon (IGBMC, Université Louis Pasteur, Illkirch, France). A pGL3basic plasmid containing 13 kb of the human CYP3A4 5'-flanking region was a kind gift of Dr. C. Liddle (University of Sydney at Westmead Hospital, Westmead, Australia). This construct was digested with *Xba*I and *Spe*I, and the resulting 343-bp fragment was further cut with *Hinc*II. The 228-bp xenobiotic-responsive enhancer module was subsequently used in electromobility shift assays and has been described (18).

Culture and Transfection of LMH Cells—Cultivation of LMH cells in Williams E medium and transfection with FuGENE 6 Transfection Reagent (Roche Molecular Biochemicals) were performed as described (16). Before transfection, cells were kept in serum-free medium for 24 h. The cells were then plated on six-well dishes, and medium was replaced 4 h after transfection by induction or control medium, respectively, both lacking fetal calf serum.

Analysis of Reporter Gene Expression—16 h after drug treatment, the cells were harvested, and nonradioactive chloramphenicol acetyltransferase (CAT) assays were performed using the CAT-ELISA kit according to the manual of the supplier (Roche Molecular Biochemicals). Cell extracts were also used for the determination of protein concentration using the ESL protein assay for normalization of specific CAT expression to total protein content (Roche Molecular Biochemicals).

Transcriptional Activation Assays—Transfection and drug treatment of CV-1 cells was performed as described (16). Cell extracts were prepared and assayed for CAT using a CAT-ELISA kit (Roche Molecular Biochemicals), and β -galactosidase activities were determined. CAT concentrations were then normalized against β -galactosidase values in order to compensate for varying transfection efficiencies.

Electromobility Shift Assays—Electromobility shift assays were performed as published (16). To test for supershifts, 0.5 μ l of either monoclonal anti-mouse RXR rabbit antibody (kindly provided by Dr. P. Chambon, IGBMC, Université Louis Pasteur, Illkirch, France) or of a

200- μ g IgG/ml anti-HA high affinity rat monoclonal antibody solution (Roche Molecular Biochemicals) were added to the reaction mix.

Amplification of Nuclear Receptors from CV-1 cDNA—CV-1 cell cDNA was used in PCRs for 40 cycles using an annealing temperature of 61.5 °C with the following primers: human CAR (5'-GAG GGC TGC AAG GGT TTC TTC AGG AGA-3' and 5'-CAG CAG GCC TAG CAA CTT CGC ATA CAG A-3'), human PXR (5'-ATC AAG CGG AAG AAA AGTGAA CGG ACA G-3' and 5'-GAG GGG CGT AGC AAA GGG GTG TAT G-3'), human LXR α (5'-CAG AGC CCC CTT CAG AAC CCA CAG AGA T-3' and 5'-GAG CAA GGC AAA CTC GGC ATC ATT GAG-3'), human LXR β (5'-CAC AGT CAC AGT CGC AGT CAC CTG-3' and 5'-GAG AAC TCG AAG ATG GGG TTG ATG AAC T-3'), human FXR (5'-GTT TCT ACC CCC AGC AGC CTG AAG AGT G-3' and 5'-CAG CGT GGT GAT TGA ATG TCC CAT A-3'), and human glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (5'-CGG GAA GCT TGT CAT CAA TGG AAA TC-3' and 5'-GCC AAA TTC GTT GTC ATA CCA GGA AAT G-3'). Bands or regions of the expected sizes (864 bp for CAR, 900 bp for PXR, 869 bp for LXR α , 532 bp for LXR β , 1134 bp for FXR, and 766 bp for GAPDH, respectively) were excised from the gel, subcloned, and sequenced.

Northern Blots—A probe for chicken CYP7A1 was amplified from chicken cDNA using degenerate primers based on the mammalian CYP7A1 sequences and verified by sequencing. A more comprehensive analysis and characterization of full-length chicken CYP7A1 mRNA will be published elsewhere. Northern hybridizations were carried out as described (16).

RESULTS

Oxysterols and Bile Acids Modify Drug Induction—In rats, CYP2B2 mRNA levels are elevated when blocking cholesterol biosynthesis using the squalene synthase inhibitor squalenstatin or the 3-hydroxy-3-methylglutaryl coenzyme A reductase inhibitor fluvastatin or lovastatin (19–21). This induction can be prevented by replenishing cholesterol levels with oxysterols. The same results were obtained with chicken CYP2H1 and CYP3A37 mRNA in the chicken hepatoma cell line LMH (22). In the CYP2H1 5'-flanking region, a 264-bp PB-responsive enhancer unit (PBRU) was isolated, and within this enhancer fragment, a DR-4 element was identified to be essential for conferring drug induction (14). We therefore tested whether inhibition of CYP2H1 induction by oxysterols is mediated by this PBRU.

As shown in Fig. 1a, both the PB and the clotrimazole induction were reduced when co-incubated with either 10 μ M 22(R)-hydroxycholesterol (22R), 24(S)-hydroxycholesterol (24S), or 20 μ M 25-hydroxycholesterol (25O), whereas none of the oxysterols affected the CYP2H1 264-bp PBRU alone. We also tested the effect of bile acids on the CYP2H1 264-bp PBRU, because bile acids are able to induce CYP2H1 and CYP3A37 mRNA (22). At 100 μ M, a concentration that physiologically occurs in bile or in cholestatic livers (23, 24), cholic acid (CA), deoxycholic acid (DCA), and chenodeoxycholic acid (CCA) all induced CAT reporter gene levels driven by the 264-bp PBRU in LMH cells (Fig. 1b). Surprisingly, co-incubation of these bile acids with PB or clotrimazole reduced the effect of the drugs (data not shown). Thus, both oxysterols and bile acids modulate drug induction of the 264-bp PBRU, comparable with their effects on CYP2H1 mRNA (22).

The 264-bp PBRU is activated by the chicken xenobiotic-sensing orphan nuclear receptor CXR (9). We therefore tested whether oxysterols or bile acids directly affect this receptor. An expression vector for GAL4(DBD)-CXR(LBD) fusion proteins together with the GAL4 upstream activating sequence (UAS) in a reporter gene vector were co-transfected into CV-1 cells, and reporter gene levels were measured after incubation with drugs, oxysterols, or bile acids. As shown in Fig. 1c, none of the oxysterols had an inhibitory effect on either PB or clotrimazole induction of the CXR-LBD. In contrast, the CXR-LBD was activated by the bile acids DCA and CCA (Fig. 1d). Apparently, the inhibition of the CYP2H1 264-bp PBRU by oxysterols is not

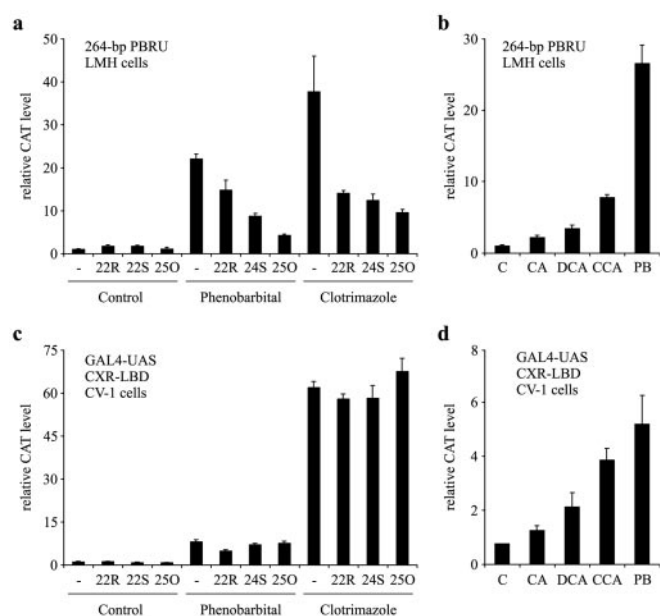


FIG. 1. Oxysterols and bile acids modulate drug induction of the CYP2H1 264-bp PBRU and of CXR-mediated transactivation. *a*, a reporter gene vector containing the chicken CYP2H1 264-bp PBRU was transfected into LMH cells cultured for 24 h in medium lacking serum. Cells were subsequently treated for 16 h with either vehicle (0.1% Me₂SO), 400 μM PB, 10 μM clotrimazole, 10 μM 22(*R*)-hydroxycholesterol (22*R*), 10 μM 24(*S*)-hydroxycholesterol (24*S*), or 20 μM 25-hydroxycholesterol (25*O*) or combinations of these compounds. The relative CAT expression was standardized against untreated control cells and expressed as -fold induction. *b*, transfected LMH cells were treated with 100 μM CA, 100 μM DCA, 100 μM CCA, or 400 μM PB. *c*, CV-1 cells were co-transfected with the GAL4(DBD)/CXR(LBD) fusion proteins and with the (UAS)5'-tk-CAT reporter gene plasmid. Cells were then treated either with vehicle (0.1% Me₂SO), 400 μM phenobarbital, 10 μM clotrimazole, 10 μM 20α-hydroxycholesterol (20α), 10 μM 22(*R*)-hydroxycholesterol (22*R*), 10 μM 24(*S*)-hydroxycholesterol (24*S*), or 20 μM 25-hydroxycholesterol (25*O*) alone or in combinations for 24 h. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. *d*, transfected CV-1 cells were treated with 100 μM CA, 100 μM DCA, 100 μM CCA, or 400 μM PB. Values are the mean of three independent experiments, and bars represent S.D.

directly mediated by CXR, whereas CXR itself constitutes a low affinity bile acid receptor.

Competition between LXR and CXR—In order to be able to test candidate receptors that might be responsible for the oxysterol and bile acid effects, we cloned the chicken LXR and chicken FXR orthologs. A cloning strategy similar to the one used for the isolation of chicken CXR was designed (9). Binding of the chicken CXR, LXR, and FXR to the CYP2H1 264-bp PBRU was examined to see if the observed effects of oxysterols and bile acids on drug induction are directly mediated by these receptors. Electromobility shift assays with radiolabeled 264-bp PBRU as probe showed that neither the chicken RXRγ, CXR, LXR, nor FXR bound alone to this enhancer element (Fig. 2*a*, lanes 2–5). Heterodimers of CXR or LXR with RXR shifted the probe (lanes 6 and 8, arrow *b*), and this complex could be supershifted when adding anti-RXR antibody (lanes 9 and 11, arrow *c*). In contrast, FXR was not able to bind to the 264-bp PBRU together with RXR (lanes 7 and 10). Thus, multiple chicken nuclear receptors are able to bind to this PBRU and others found in the CYP2H1 5'-flanking region (15).

Based on the observed LXR interaction with the CYP2H1 264-bp PBRU, we examined whether CXR and LXR bind to the same DR-4 element within this PBRU that has previously been shown to be responsible for CYP induction by CXR (9). In electromobility shift assays, CXR/RXR heterodimers bound strongly to the radiolabeled, wild type 264-bp PBRU and much

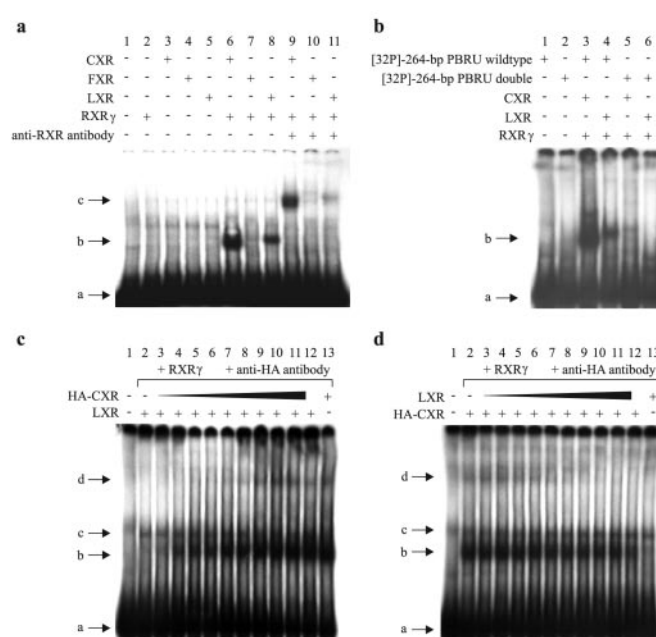


FIG. 2. CXR and LXR, but not FXR, compete for binding to the same DR-4 element within the CYP2H1 264-bp PBRU. *a*, radiolabeled 264-bp PBRU was incubated with *in vitro* transcribed/translated CXR (lanes 3, 6, and 9), FXR (lanes 4, 7, and 10), LXR (lanes 5, 8, and 11), chicken RXRγ (lanes 2 and 6–11), and anti-RXR antibody (lanes 9–11). The arrows depict the unbound probe (arrow *a*), the complex of CXR and LXR with RXRγ (arrow *b*), and the supershift of these complexes after the addition of anti-RXR antibody (arrow *c*). *b*, radiolabeled wild type 264-bp PBRU (wildtype, lanes 1, 3, and 4) or radiolabeled 264-bp PBRU with a double mutation in the DR-4 site (double, lanes 2, 5, and 6) were incubated with *in vitro* transcribed/translated CXR (lanes 3 and 5), LXR (lanes 4 and 6), and chicken RXRγ (lanes 3–6). The arrows depict the unbound probe (*a*) and the complex of CXR and LXR with RXRγ (*b*). *c*, radiolabeled wild type 264-bp PBRU was incubated with *in vitro* transcribed/translated CXR containing an N-terminal hemagglutinin tag (HA-CXR) (lanes 3–13), chicken LXR (lanes 2–12), chicken RXRγ (lanes 2–13), and anti-HA antibody (lanes 2–13) as indicated. Increasing HA-CXR concentrations were applied from lane 3 to lane 12. The arrows depict the unbound probe (arrow *a*), the complex of HA-CXR with RXRγ (arrow *b*), the shift of the LXR/RXRγ complex bound to the 264-bp PBRU (arrow *c*), and the supershift of HA-CXR and RXRγ together with anti-HA antibody (arrow *d*). *d*, electromobility shifts with increasing concentrations of LXR from lane 3 to lane 13 and constant concentrations of HA-CXR in lanes 2–12.

more weakly to the radiolabeled 264-bp PBRU containing mutations in both hexamer half-sites of the DR-4 element (Fig. 2*b*, lanes 3 and 5, arrow *b*). Similarly, LXR/RXR heterodimers only bound to the wild type 264-bp PBRU but not to the DR-4 mutant (lanes 4 and 6). Apparently, both CXR and LXR heterodimerize with RXR and bind to the same sequence elements on the 264-bp PBRU.

Since LXR and CXR bind to the same DR-4 element, electromobility shift assays were used to elucidate if LXR and CXR directly compete for binding to this PBRU. To clearly discriminate between complexes containing CXR or LXR, an HA tag was N-terminally attached to CXR, and an anti-HA monoclonal antibody was used to supershift complexes that include HA-CXR. Constant LXR concentrations were titrated against increasing concentrations of HA-CXR with chicken RXR and anti-HA antibody included in all reactions (Fig. 2*c*, lanes 3–12). With increasing HA-CXR levels, a shift that is lower compared with the LXR/RXR shift (lane 2, arrow *c*) and a supershift became gradually visible, and the LXR/RXR shift decreased correspondingly (lanes 3–12, arrow *b* for the HA-CXR/RXR shift, arrow *c* for the LXR/RXR shift and arrow *d* for the supershift). The lower shift (arrow *b*) and the supershift (arrow *d*) were also observed in a control reaction with HA-CXR and

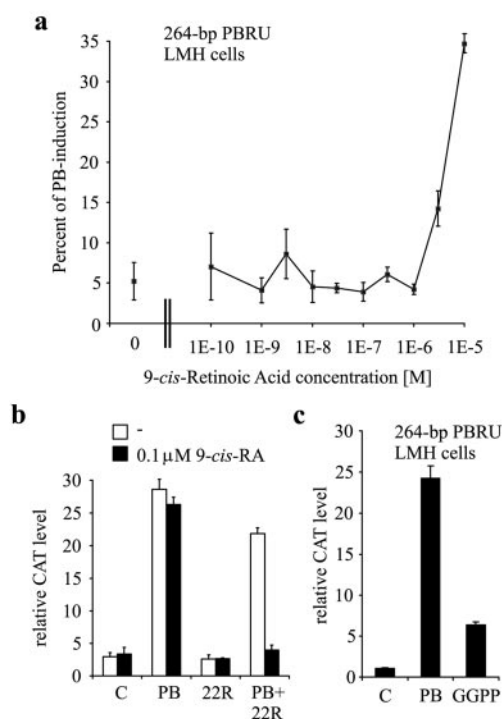


FIG. 3. Oxysterols and 9-cis-retinoic acid synergistically inhibit drug induction of the CYP2H1 264-bp PBRU. *a*, the chicken CYP2H1 264-bp PBRU was transfected into LMH cells that had been cultured for 24 h in medium without serum. Cells were subsequently treated for 16 h with either vehicle (0.1% Me₂SO), 400 μM PB, or increasing concentrations of 9-cis-retinoic acid (0.1 nM to 10 μM). Cells were harvested, and CAT levels were determined. The relative CAT expression was standardized against PB-treated cells and expressed as a percentage of PB induction. *b*, LMH cells transfected with the 264-bp PBRU were treated for 16 h with vehicle, 400 μM PB, 10 μM 22(*R*)-hydroxycholesterol (22*R*), 0.1 μM 9-cis-retinoic acid (9-cis-RA), or combinations of these drugs. The relative CAT expression was standardized against untreated cells and expressed as -fold induction. Values are the mean of three independent experiments, and bars represent S.D. *c*, transfected LMH cells were treated with 400 μM PB or a 25 μM concentration of the LXR inhibitor GGPP for 16 h before CAT levels were measured.

RXR (lane 13). *Vice versa*, in electromobility shifts using constant amounts of HA-CXR and increasing levels of LXR, the HA-CXR/RXR supershift was gradually reduced (Fig. 2*d*). These results imply that LXR and CXR directly compete for heterodimerization with RXR and subsequent binding to the DR-4 element in the CYP2H1 264-bp PBRU. It is realized that *in vitro* electromobility shift assays do not allow a conclusion about the relative affinities of the different nuclear receptor heterodimers to the DNA-binding sites *in vivo*. However, these experiments demonstrate that both LXR/RXR and CXR/RXR heterodimers bind to the same DR-4 sites and that, by changing the concentration of one of the components, changes in the binding of the rival complex can be observed.

Activators of LXR and RXR Synergistically Inhibit PB Induction—Functional evidence for the inhibitory action by LXR was also obtained by experiments in LMH cells transfected with the 264-bp PBRU using varying concentrations of 9-cis-retinoic acid. After 16 h of treatment, the 264-bp PBRU was only activated by micromolar concentrations of 9-cis-retinoic acid, much more than required to activate RXR in permissive nuclear receptor heterodimers (Fig. 3*a*). This suggests that CXR is nonpermissive like PXR and CAR (25). In LMH cells transfected with the 264-bp PBRU, 10 μM 22(*R*)-hydroxycholesterol (22*R*) or 0.1 μM 9-cis-retinoic acid (9-cis-RA) only marginally change reporter gene levels after 16 h (Fig. 3*b*). In striking contrast, the combination of 22(*R*)-hydroxycholesterol

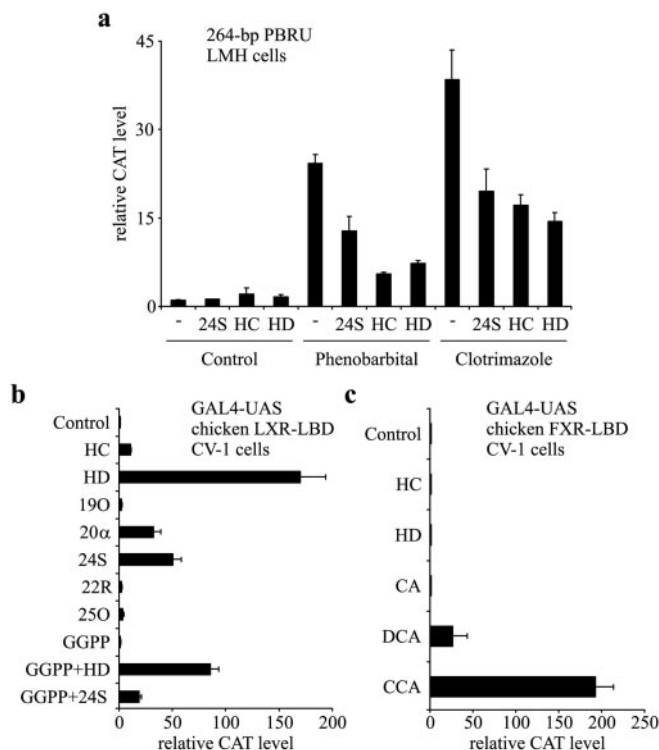


FIG. 4. Hydroxylated bile acids repress drug induction of the CYP2H1 264-bp PBRU and activate LXR. *a*, the chicken CYP2H1 264-bp PBRU was transfected into LMH cells that had been cultured for 24 h in medium lacking serum. Cells were subsequently treated for 16 h with either vehicle (0.1% Me₂SO), 400 μM PB, 10 μM clotrimazole, 10 μM 24(*S*)-hydroxycholesterol (24*S*), 10 μM HC, 10 μM HD, or combinations of these compounds. The relative CAT expression was standardized against untreated cells and expressed as -fold induction. *b* and *c*, CV-1 cells were co-transfected with the GAL4(DBD)-LXR(LBD) (Fig. 4*b*) or the GAL4(DBD)-FXR(LBD) (Fig. 4*c*) fusion proteins, respectively, together with the reporter gene plasmid (UAS)⁵-tk-CAT. Cells were subsequently treated either with vehicle (0.1% Me₂SO), 10 μM 19-hydroxycholesterol (19*O*), 10 μM 20α-hydroxycholesterol (20α), 10 μM 24(*S*)-hydroxycholesterol (24*S*), 10 μM 22(*R*)-hydroxycholesterol (22*R*), 20 μM 25-hydroxycholesterol (25*O*), 10 μM CA, 10 μM CCA, 10 μM HC, 10 μM HD, 25 μM GGPP, or combinations of these drugs for 24 h. Cell extracts were analyzed for CAT expression normalized against β-galactosidase levels. Values represent the mean of three independent experiments, and bars represent S.D.

and 9-cis-retinoic acid synergistically inhibits drug induction of the 264-bp PBRU (Fig. 3*b*).

Further proof for the involvement of LXR was obtained by treating LMH cells with 25 μM geranylgeranyl-pyrophosphate (GGPP), an inhibitor of LXRα that reduces the interaction between LXRα and the nuclear receptor co-activator SRC-1 (26, 27). GGPP was able to induce the CYP2H1 264-bp PBRU after a 16-h treatment (Fig. 3*c*). These results strongly suggest that the permissive oxysterol receptor LXR (28) is responsible for the oxysterol-mediated inhibition of drug induction. Moreover, these results suggest that LXR might inhibit xenosensor-mediated drug induction by other mechanisms in addition to mere competition for binding to the DR-4 site.

Hydroxylated Bile Acids Activate LXR—In mammals, CYP3As and to a lesser extent CYP2Cs and CYP2Bs are capable of hydroxylating bile acids (29, 30). Moreover, a specific subset of hydroxylated bile acids were shown to induce both LXRα and LXRβ in transactivation assays (31). Assuming that chicken CYP2H1 is also involved in bile acid hydroxylation, we tested the effect of 6α-hydroxylated CCA (hyocholic acid (HC)) and 6α-hydroxylated lithocholic acid (hyodeoxycholic acid (HD)) on drug induction of the CYP2H1 264-bp PBRU and on activation of chicken LXR and FXR, respectively. As depicted in

Fig. 4a, 10 μM HC or HD had no effect on the CYP2H1 264-bp PBRU alone, but both compounds severely reduced PB and clotrimazole induction comparable with 10 μM 24(S)-hydroxycholesterol (24S) after 16-h induction in LMH cells.

In CV-1 cell transactivation assays with the GAL4(DBD)-LXR(LBD) and GAL4(DBD)-FXR(LBD) fusion proteins and the GAL4-response element UAS in a reporter gene vector, HC and HD activated the chicken LXR LBD (Fig. 4b). In contrast, chicken FXR LBD was not affected by hydroxylated bile acids after a 24-h incubation at a dose of 10 μM (Fig. 4c). As control compounds, the oxysterols 20 α -hydroxycholesterol (Fig. 4b, 20 α (10 μM)) and 24(S)-hydroxycholesterol (24S, 10 μM) strongly activated chicken LXR. The oxysterols 19-hydroxycholesterol (19O, 10 μM), 22(R)-hydroxycholesterol (22R, 10 μM), and 25-hydroxycholesterol (25O, 20 μM) showed relatively small effects (Fig. 4b). Moreover, 25 μM GGPP inhibited both 24(S)-hydroxycholesterol- and hyodeoxycholic acid-mediated induction of LXR (Fig. 4b). DCA and CCA markedly activated the chicken FXR construct GAL4(DBD)-FXR(LBD) in CV-1 cell transactivation assays, whereas cholic acid (CA) had no effect (Fig. 4c). Thus, chicken LXR and FXR exhibit similar activation patterns as their mammalian orthologs (3). These findings suggest that hydroxylated bile acid-mediated activation of chicken LXR is responsible for the inhibition of the CYP2H1 264-bp PBRU.

Human LXR α Competes with PXR and CAR—Having established the cross-talk between LXR and the xenobiotic-sensing orphan nuclear receptor CXR in chicken, we wanted to know whether a corresponding regulatory mechanism of LXR α competing with PXR and CAR exists in humans. Accordingly, electromobility shift assays with wild type and mutated radiolabeled human CYP3A4 xenobiotic-responsive enhancer module (18) and CYP2B6 51-bp PBREM (32) showed specific binding of human PXR, human CAR, and human LXR α , each of these receptors heterodimerized with RXR α . This binding was only observed when using the wild type probe but not with mutated probe, as shown for the CYP2B6 PBREM (Fig. 5a, lanes 2–7 and lanes 9–14).

PCR amplifications of CV-1 cDNA revealed the presence of LXR α , LXR β , and FXR in this monkey kidney epithelial cell line, whereas expression of neither PXR nor CAR could be detected (data not shown). To establish a direct role of LXR in mediating the inhibitory effect, we transfected the CV-1 cells with increasing concentrations of LXR and measured activation of the chicken CYP2H1 264-bp PBRU (Fig. 5b). After co-transfecting the CYP2H1 264-bp PBRU, chicken LXR, and CXR, CV-1 cells were treated with either vehicle, 20 μM 25-hydroxycholesterol (Fig. 5b, 25O), 10 μM clotrimazole (*clo*) or combinations of these compounds. Increasing concentrations of co-transfected LXR decreased the reporter gene expression controlled by the PBRU both without and with clotrimazole as an inducer (Fig. 5b). The same effects were seen by using 10 μM 22(R)-hydroxycholesterol instead of 25-hydroxycholesterol (data not shown). These results imply a direct role of LXR in the inhibition of the PBRU. Since the CV-1 cells express endogenous LXR, co-transfection of additional LXR was not needed to test the effect of oxysterols on activation of full-length human PXR, human CAR, or chicken CXR in CV-1 transactivation assays. As depicted in Fig. 5c, PXR-triggered activation of a CYP3A4 PXR-responsive ER-6 element that also contains a DR-4 element (10) and the CYP2B6 51-bp PBREM by 400 μM PB could be prevented in co-incubation experiments with 20 μM 25-hydroxycholesterol. In the same set of experiments, CAR could not be activated by PB, but its basal activity was decreased by 25-hydroxycholesterol on the CYP3A4 ER-6 and the CYP2B6 51-bp PBREM (Fig. 5d). As a control, CXR activation of the CYP2H1 264-bp PBRU by PB was also inhibited

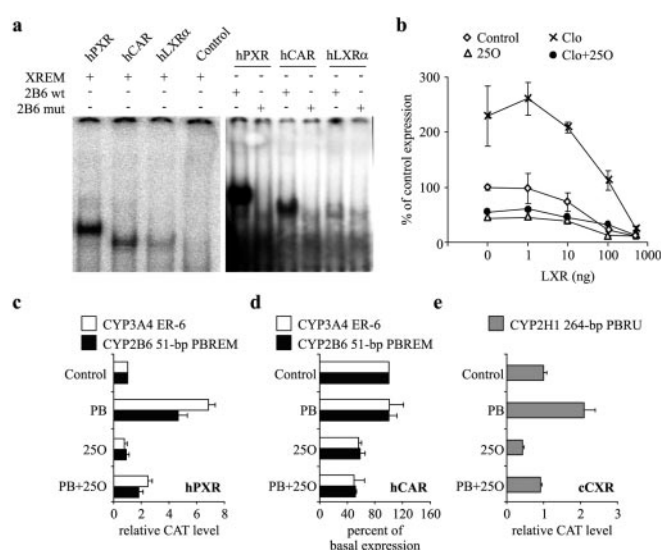


FIG. 5. Human LXR α inhibits drug activation of the human CYP3A4 and CYP2B6 PB-responsive enhancer elements by human PXR and CAR. *a*, radiolabeled wild type xenobiotic-responsive enhancer module of human CYP3A4 or 51-bp PBREM of CYP2B6 as well as the corresponding PBREM sequence containing a double mutation in the DR-4 sequence were incubated with either mock transcribed/translated reticulocyte lysate or with *in vitro* transcribed/translated human PXR, CAR, or LXR α together with RXR α . *b*, the inhibition of the PBRU is LXR-dependent. CV-1 cells were co-transfected with the chicken CYP2H1 264-bp PBRU, CXR, and increasing doses of chicken LXR. After transfection, cells were treated for 24 h with either vehicle, 20 μM 25-hydroxycholesterol (25O), 10 μM clotrimazole (*clo*), or combinations of these compounds. Cells were harvested, and reporter gene expression levels were analyzed and normalized against β -galactosidase levels. *c–e*, CV-1 cells were co-transfected with either human PXR (*c*), human CAR (*d*), or chicken CXR (*e*) together with the CYP3A4 ER-6, CYP2B6 51-bp PBREM, and the CYP2H1 264-bp PBRU, respectively. After treatment with vehicle (0.1% Me₂SO), 400 μM PB, 10 μM 25-hydroxycholesterol (25O), or combinations of these chemicals for 24 h, cell extracts were analyzed for CAT expression normalized against β -galactosidase levels. Values are the mean of three independent experiments, and bars represent S.D.

by 25-hydroxycholesterol (Fig. 5e), suggesting that the cross-talk between LXR and xenobiotic-sensing receptors is a common mechanism conserved from birds to humans.

Phenobarbital Represses Expression of Chicken CYP7A1—Our results thus demonstrate an antagonistic effect of the cholesterol sensor LXR and the xenosensors PXR, CAR, and CXR on the expression of drug-induced CYPs. Inversely, LXR up-regulates mRNA levels of CYP7A1, whereas several findings suggest a PXR-dependent repression of CYP7A1 by Cyp3a inducers in mouse (33). Accordingly, we tested whether the diametrically opposed effects of LXR and xenosensors are also observed in chicken. Total RNA from LMH cells treated for 24 h with either vehicle, 400 μM PB, 20 μM 25-hydroxycholesterol, or 100 μM CCA was isolated and subjected to Northern hybridization using probes against chicken CYP7A1 and chicken GAPDH. As shown in Fig. 6, chicken CYP7A1 expression levels are markedly reduced both in LMH cells treated with bile acids and with PB correlating well with the results found in mammals with PXR activators. Interestingly, chicken CYP7A1 mRNA is neither induced by 25-hydroxycholesterol (Fig. 6) nor by 24(S)-hydroxycholesterol (data not shown), unlike rodent Cyp7a1 but similar to human CYP7A1 (34).

DISCUSSION

In the present study, a regulatory interaction between endogenous cholesterol and bile acid homeostasis signaling pathways and drug-mediated induction of CYPs is established. Our data show that the oxysterol sensor LXR controls activation of

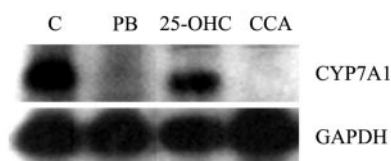


FIG. 6. Chicken CYP7A1 expression is inhibited by bile acids and phenobarbital. Total RNA from LMH cells treated with vehicle, 400 μM PB, 20 μM 25-hydroxycholesterol (25-OHC), or 100 μM CCA was isolated, and 20 μg were analyzed on Northern blots using radiolabeled probes designed from the chicken CYP7A1 and the chicken GAPDH cDNA sequences, respectively.

drug-sensitive enhancer elements by interacting with the xenobiotic-sensing orphan nuclear receptors CXR, PXR, and CAR. These receptors compete by inhibiting or activating drug-activated enhancer elements, respectively. Our findings therefore indicate a direct molecular link between hepatic cholesterol levels and drug or xenobiotic induction of CYPs.

A significant part of hepatic cholesterol is metabolized to bile acids. Bile acids are important regulators of cholesterol homeostasis by inhibiting hepatic cholesterol metabolism into bile acids or by enhancing uptake of dietary cholesterol. Thus, the levels of bile acids and cholesterol are linked and tightly controlled. This link occurs at the level of transcriptional regulation of CYP7A1 via the positively acting oxysterol-receptor LXR in rodents and is opposed by the negative effect of bile acids and the bile acid receptor FXR (7, 8) (Fig. 7a). Under pathological conditions such as cholestasis, bile acids accumulate in the liver and cause cell damage. Additional mechanisms are needed under these conditions for bile acid metabolism and excretion. Here, we show that the xenobiotic-sensing nuclear receptor CXR is a low affinity bile acid receptor and is therefore capable of inducing CYP2H1 and CYP3A37 in the presence of high bile acid levels in a chicken hepatoma cell line. In mice and humans, the xenosensor PXR is also activated by high bile acid levels and plays a role in prevention of bile acid-induced hepatotoxicity (13, 17). Thus, when bile acids accumulate in the liver and reach toxic concentrations, they activate xenobiotic-sensing nuclear receptors and stimulate their own metabolism into more hydrophilic hydroxylated bile acids, which are renally excreted. This concept has recently been demonstrated in the FXR-null mouse where bile acid export into bile is reduced and thus leads to elevated hepatic bile acid levels (35). Strikingly, hydroxylated bile acids inhibit drug activation of drug- and bile acid-metabolizing CYPs and therefore directly regulate their own levels in the liver. In this report, we could show that hydroxylated bile acids activate the oxysterol-sensor LXR. Therefore, the inhibitory effects of oxysterols and hydroxylated bile acids are mediated by the same mechanism. Both in chickens and in mice (3, 13), drugs activating PXR or CXR negatively affect the transcript level of CYP7A1, thereby inhibiting the biosynthesis of bile acids from cholesterol but also potentially elevating plasma cholesterol levels. Consequently, we propose a novel regulatory mechanism by which the levels of cholesterol, bile acids, and hydroxylated bile acids in the liver are regulated by both drug-activated transcription factors and the oxysterol-sensing nuclear receptors (Fig. 7b). Of course, although not depicted in Fig. 7b, LXR activation by oxysterols potentially leads to inhibition of drug-metabolizing CYPs as LXR activation by hydroxylated bile acids might elevate Cyp7a1 levels in rodents. In conjunction with drug- and bile acid-metabolizing CYPs, phase II enzymes and transporters are activated by xenosensors, which therefore control a whole enzyme battery for metabolism and clearance of high levels of lipophilic, toxic compounds (13, 36, 37).

This is the first report describing inhibition of gene expression by LXR/RXR heterodimers on a DR-4 element. The mechanistic explanation for this inhibition has not been elucidated yet and is under current investigation. Our results suggest that apart from

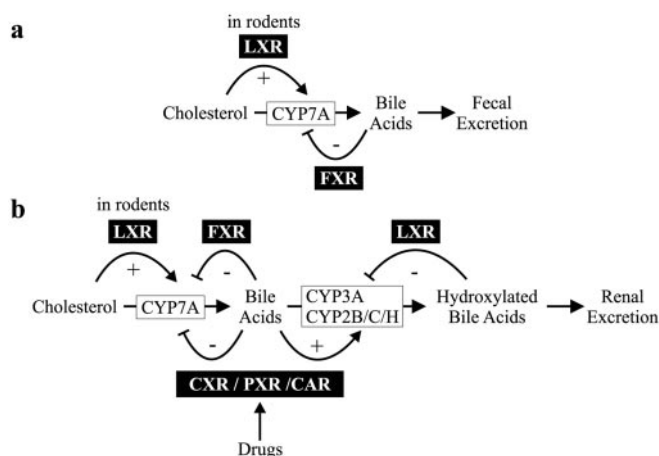


FIG. 7. Proposed regulatory interplay between drug-metabolizing CYPs and cholesterol as well as bile acid homeostasis under normal and pathological conditions. *a*, under normal conditions, cholesterol controls its metabolism to bile acids by activating LXR in rodents. High bile acid levels consequently reduce this pathway by inhibiting CYP7A1, the first and rate-limiting enzyme in bile acid biosynthesis. This inhibition is dependent on FXR. Bile acids are predominantly excreted via bile and feces. *b*, when bile acids accumulate in the liver, they also activate the drug-sensing nuclear receptors CXR in chicken as well as PXR and potentially CAR in mammals. Subsequently, bile acids are hydroxylated by CYPs of the subfamilies CYP3A/2B/2C and 2H and can then be excreted via blood and urine. High levels of hydroxylated bile acids activate LXR, which competes with the xenosensors CXR, PXR, and CAR for binding to enhancer elements in the 5'-flanking regions of these CYPs. PXR, CXR, and CAR also inhibit formation of bile acids by negative regulation of CYP7A1 by so far unknown mechanisms.

direct competition for binding to the same recognition sites, additional LXR-dependent mechanisms might play a role in the inhibition of drug-induced PBRU activation. Moreover, the results reported here have been obtained in *in vitro* assays such as cell culture or electromobility shift assays. Verification of the hypothesis of LXR-CXR/PXR/CAR cross-talk in drug-inducible CYP induction is currently being studied using mouse models with deficiencies in the respective receptors. However, our hypothesis is supported by numerous *in vivo* experimental and clinical observations. As examples, treatment with the inhibitors of cholesterol biosynthesis squalastatin, lovastatin, or fluvastatin induces CYP2B1/2 in primary rat hepatocytes or rat liver *in vivo* (19–21). Rats fed a high cholesterol diet or spontaneous hyperlipidemic rats with 3–4-fold increased cholesterol levels have lower expression of basal and PB-induced CYPs compared with control animals (38, 39) and exhibit changes in the expression of several enzymes encoding cholesterol-synthesizing and metabolizing enzymes (40, 41). Obese fa/fa Zucker rats fail to exhibit a significant induction response of CYP2B1/2 after PB treatment (42). PB treatment of epileptic patients or of rats resulted in increased plasma cholesterol and lipoprotein levels (43–48). Similarly, human immunodeficiency virus-protease inhibitor therapy in AIDS patients often leads to elevated cholesterol and triglyceride levels. Among the current protease inhibitors, ritonavir is associated with the highest frequency of hypercholesterolemia in contrast to saquinavir, indinavir, or nelfinavir, which are reported to have markedly lower relative risks for hypercholesterolemia (49). Recently, ritonavir has been shown to bind to and activate human PXR, whereas saquinavir is a weak activator, and nelfinavir and indinavir do not affect PXR at all (50). The present results now offer an explanation for these clinical observations.

Under normal conditions and in cholestasis, CCA and hyocholic acid, respectively, belong to the major components of bile in human and rat hepatocytes (23, 24). Moreover, hyocholic and

hydroxycholelic acid are also found in the serum and urine of cholestatic patients treated with PB or rifampicin (51–53). For years, cholestatic patients have been empirically treated with PB or rifampicin without knowing the molecular mechanism underlying the beneficial effect (51, 54). The antagonistic effect of oxysterol-activated LXR on drug-induced CYPs by the xenobiotic-sensing nuclear receptors CXR, CAR, and PXR and the activation of these transcription factors by bile acids described in this report contribute to our understanding of the mechanism underlying the clinical remission of cholestatic symptoms in patients after drug treatment. Metabolic disorders affecting cholesterol homeostasis such as hypercholesterolemia or hypertriglyceridemia are prevalent in industrialized countries and are associated with serious diseases like atherosclerosis, cardiovascular disorders, or adult onset diabetes. The nuclear receptors LXR and FXR are attractive new drug targets for treatment of some of these diseases (55). For example, activation of LXR in macrophages has been described to reduce atherosclerosis and plasma low density lipoprotein levels by inducing the transcription of ABC-type transporters and apolipoprotein E (56, 57). We and others (31) found that a subset of hydroxylated bile acids activate LXR. Accordingly, administered hyocholic acid efficiently suppresses atherosclerosis formation and lowers plasma cholesterol levels in mice (58). Nevertheless, due to the cross-talk of these receptors with the hepatic detoxification system, potential adverse drug reactions have to be considered (e.g. in cholesterol-lowering therapies using a combination of LXR agonists, which decrease the low density lipoprotein part and increase the high density lipoprotein part of the cholesterol together with statins that lower *de novo* biosynthesis).

Our studies thus support the concept that the molecular mechanism of hepatic drug induction is closely linked to endogenous regulatory pathways. This leads to speculations about the evolutionary origin of drug-metabolizing CYPs and xenobiotic-sensing nuclear receptors. Many of the drug-metabolizing CYPs also catalyze the biotransformation of steroid hormones and bile acids. Long term treatment with inducer compounds drastically alters steroid metabolism and elevates steroid clearance (59). The findings reported here also demonstrate an influence of cholesterol and bile acid levels on hepatic drug metabolism. Certain bile acids activate the detoxification system, whereas other cholesterol metabolites such as oxysterols or hydroxylated bile acids reduce the corresponding CYP expression. The system of these nuclear receptors and CYPs probably evolved to handle accumulated toxic cholesterol metabolites that have detergent properties. Later, both these nuclear receptors and the CYPs may have extended their substrate specificity to include xenobiotic compounds with similar hydrophobic properties. Seemingly, our body deals with drugs and other xenobiotics by handling them as “toxic bile acids.”

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Cholesterol and Bile Acids Regulate Xenosensor Signaling in Drug-mediated Induction of Cytochromes P450

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