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Summary

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 (PXR), constitutive androstane receptor (CAR) 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 (LXR) competes with CXR, PXR or CAR 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.

Introduction

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 halfsites 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 (SHP) 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 (SXR) or pregnane-activated receptor (PAR), and chicken xenobiotic receptor (CXR) were discovered to be involved in drug-induction of CYP2Bs, CYP3As and CYP2H1 in man, mouse and chicken, 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 chicken, 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). In spite of 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 man (9,14-16).

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 acids (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 suggests 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 ($\text{RXR}\gamma$), FXR and LXR were amplified and subcloned into the expression vector pSG5 (Stratagene, Basel, Switzerland). Chicken CXR (amino acids 97-391), FXR (amino acid 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 EcoRI-site of the pSG5-CXR expression vector. The (UAS)5-tk-CAT reporter plasmid was generously provided by Dr. S. A. Kliewer, Department of Molecular Endocrinology, GlaxoSmithKline Research and Development, Research Triangle Park, NC. Oligonucleotides for the wildtype CYP2B6 51-bp PB-responsive enhancer module (PBREM) and the corresponding 51-bp where the hexamer halvesites of the two DR-4 elements were mutated into SacII and EcoRV sites, respectively, were synthesized by Microsynth, Balgach, Switzerland. Similarly, a wildtype 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 XbaI and SpeI and the resulting 343-bp fragment further

cut with HincII. The 228-bp xenobiotic-responsive enhancer module (XREM) was subsequently used in electromobility shift assays and has been described (18).

Culture and Transfection of LMH Cells.

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

Analysis of Reporter Gene Expression.

16 hours after drug treatment, the cells were harvested and non-radioactive chloramphenicol acetyltransferase (CAT) assays were performed using the CAT-ELISA kit according to the manual of the supplier (Roche Molecular Biochemicals, Rotkreuz, Switzerland). 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, Rotkreuz, Switzerland).

Transcriptional activation assays.

Transfection and drug treatment of CV-1 cells was performed as described (16). Cell extracts were prepared, assayed for CAT using a CAT-ELISA kit (Roche Molecular Biochemicals, Rotkreuz, Switzerland) 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, Rotkreuz, Switzerland) were added to the reaction mix.

Amplification of nuclear receptors from CV-1 cDNA.

CV-1 cell cDNA was used in PCR reactions 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 GAT TGA ATG TCC GTA 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 rat, CYP2B2 mRNA levels are elevated when blocking cholesterol biosynthesis using the squalene synthase inhibitor squalenstatin or the 3-hydroxy-3-methylglutaryl co-enzyme A reductase inhibitors 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 if 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, concentrations that physiologically occur 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 to 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 if 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 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 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 RXR γ , CXR, LXR nor FXR bound alone to this enhancer element (Fig. 2a, 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, wildtype 264-bp PBRU and much weaker to the radiolabeled 264-bp PBRU containing mutations in both hexamer half-sites of the DR-4 element (Fig. 2b, lanes 3 and 5, arrow b). Similarly, LXR/RXR heterodimers only bound to the wildtype

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, a 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. 2c, lanes 3-12). With increasing HA-CXR levels, a shift that is lower compared to 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 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. 2d). 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.

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 hours 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. 3a). This suggests that CXR is non-permissive like PXR and CAR (25). In LMH cells transfected with the 264-bp PBRU, 10 μ M 22(R)-hydroxycholesterol (22R) or 0.1 μ M 9-cis-retinoic acid (9-cis-RA) only marginally change reporter gene levels after 16 hours (Fig. 3b). In striking

contrast, the combination of 22(R)-hydroxycholesterol and 9-cis-retinoic acid synergistically inhibits drug-induction of the 264-bp PBRU (Fig. 3b).

Further proof for the involvement of LXR was obtained by treating LMH cells with 25 μ M geranylgeranyl-pyrophosphate (GGPP), an inhibitor of LXR α that reduces the interaction between LXR α and the nuclear receptor coactivator SRC-1 (26,27). GGPP was able to induce the CYP2H1 264-bp PBRU after a 16 hours treatment (Fig. 3c). These results strongly suggest that the permissive oxysterol-receptor LXR (28) is responsible for the oxysterol-mediated inhibition of drug-induction.

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 LXR α and LXR β in transactivation assays (31). Assuming that chicken CYP2H1 is also involved in bile acid hydroxylation, we tested the effect of 6 α -hydroxylated CCA (hyocholic acid, HC) and 6 α -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 μ M hyocholic acid (HC) or hyodeoxycholic acid (HD) had no effect on the CYP2H1 264-bp PBRU alone but both compounds severely reduced PB- and clotrimazole-induction comparable to 10 μ M 24(S)-hydroxycholesterol (24S) after 16 hours 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, hyocholic acid (HC) and hyodeoxycholic acid (HD) activated the chicken LXR LBD (Fig. 4b). In contrast, chicken FXR LBD was not affected by hydroxylated bile acids after 24 hours incubation at a dose of 10 μ M (Fig. 4c). As control compounds, the oxysterols 20 α -hydroxycholesterol

(20 α , 10 μ M) and 24(S)-hydroxycholesterol (24S, 10 μ M) strongly activated chicken LXR. The oxysterols 19-hydroxycholesterol (19O, 10 μ M), 22(R)-hydroxycholesterol (22R, 10 μ M) and 25-hydroxycholesterol (25O, 20 μ M) showed relatively small effects (Fig. 4b). Moreover, 25 μ M GGPP inhibited both 24(S)-hydroxycholesterol- and hyodeoxycholic acid-mediated induction of LXR (Fig. 4b). Deoxycholic acid (DCA) and chenodeoxycholic acid (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 crosstalk 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 man. Accordingly, electromobility-shift assays with wildtype and mutated radiolabeled human CYP3A4 XREM (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 wildtype 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 (Fig. 5b, lanes 3-5) whereas expression of neither PXR nor CAR could be detected (lanes 1 and 2). Therefore, 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 because the same results were obtained with and without co-transfecting LXR (data not shown). 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 μ M PB could be prevented in co-incubation experiments with 20 μ M 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 by 25-hydroxycholesterol (Fig. 5e) suggesting that the crosstalk between LXR and xenobiotic-sensing receptors is a common mechanism conserved from birds to man.

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 upregulates mRNA levels of CYP7A1 whereas several findings suggest a PXR-dependent repression of CYP7A1 by Cyp3a inducers in mouse (33). Accordingly, we tested if the diametrically opposed effects of LXR and xenosensors is also observed in chicken. Total RNA from LMH cells treated for 24 hours with either vehicle, 400 μ M PB, 20 μ M 25-hydroxycholesterol (25-OHC) or 100 μ M 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 drug-sensitive enhancer elements by competing with the xenobiotic-sensing orphan nuclear receptors CXR, PXR and CAR. These receptors compete activating or inhibiting 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 mouse and man, 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 chicken and in mouse (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 mechanisms 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). 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. 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 crosstalk 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 to control animals (38,39) and exhibit changes 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, HIV-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 to 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 hyodeoxycholic 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 now provides an explanation for 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 LDL-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 crosstalk 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 LDL- and increase the HDL-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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The chicken FXR and LXR sequences have been deposited in GenBank (accession numbers XXX and XXX, respectively).

¹Abbreviations

CYP, cytochrome P-450; PB, phenobarbital; LXR, liver X receptor; FXR, farnesoid X receptor; SHP, small heterodimer partner; CAR, constitutive androstane receptor; PXR, pregnane X receptor; SXR, steroid and xenobiotic receptor; PAR, pregnane activated receptor; CXR, chicken xenobiotic receptor; RXR, 9-cis retinoid acid receptor; LBD, ligand-binding domain; PBREM, PB-responsive enhancer module; XREM, xenobiotic-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, geranyl-geranyl pyrophosphate

References

1. Waxman, D. J., and Azaroff, L. (1992) *Biochem J* **281**(Pt 3), 577-92
2. Nelson, D. R. (1999) *Arch Biochem Biophys* **369**(1), 1-10
3. Repa, J. J., and Mangelsdorf, D. J. (2000) *Annu Rev Cell Dev Biol* **16**, 459-81
4. Honkakoski, P., and Negishi, M. (2000) *Biochem J* **347**(Pt 2), 321-37
5. Waxman, D. J. (1999) *Arch Biochem Biophys* **369**(1), 11-23
6. Savas, U., Griffin, K. J., and Johnson, E. F. (1999) *Mol Pharmacol* **56**(5), 851-7
7. Goodwin, B., Jones, S. A., Price, R. R., Watson, M. A., McKee, D. D., Moore, L. B., Galardi, C., Wilson, J. G., Lewis, M. C., Roth, M. E., Maloney, P. R., Willson, T. M., and Kliewer, S. A. (2000) *Mol Cell* **6**(3), 517-26
8. Lu, T. T., Makishima, M., Repa, J. J., Schoonjans, K., Kerr, T. A., Auwerx, J., and Mangelsdorf, D. J. (2000) *Mol Cell* **6**(3), 507-15
9. Handschin, C., Podvinec, M., and Meyer, U. A. (2000) *Proc Natl Acad Sci U S A* **97**(20), 10769-74
10. Xie, W., Barwick, J. L., Simon, C. M., Pierce, A. M., Safe, S., Blumberg, B., Guzelian, P. S., and Evans, R. M. (2000) *Genes Dev* **14**(23), 3014-23
11. Wei, P., Zhang, J., Egan-Hafley, M., Liang, S., and Moore, D. D. (2000) *Nature* **407**(6806), 920-3
12. Xie, W., Barwick, J. L., Downes, M., Blumberg, B., Simon, C. M., Nelson, M. C., Neuschwander-Tetri, B. A., Brunt, E. M., Guzelian, P. S., and Evans, R. M. (2000) *Nature* **406**(6794), 435-9
13. Staudinger, J. L., Goodwin, B., Jones, S. A., Hawkins-Brown, D., MacKenzie, K. I., LaTour, A., Liu, Y., Klaassen, C. D., Brown, K. K., Reinhard, J., Willson, T. M., Koller, B. H., and Kliewer, S. A. (2001) *Proc Natl Acad Sci U S A* **98**(6), 3369-74
14. Handschin, C., and Meyer, U. A. (2000) *J Biol Chem* **275**(18), 13362-9

15. Handschin, C., Podvinec, M., Looser, R., Amherd, R., and Meyer, U. A. (2001) *Mol Pharmacol* **60**(4), 681-9
16. Handschin, C., Podvinec, M., Stöckli, J., Hoffmann, K., and Meyer, U. A. (2001) *Mol Endocrinol* **15**(9), 1571-85
17. Xie, W., Radominska-Pandya, A., Shi, Y., Simon, C. M., Nelson, M. C., Ong, E. S., Waxman, D. J., and Evans, R. M. (2001) *Proc Natl Acad Sci U S A* **98**(6), 3375-80
18. Goodwin, B., Hodgson, E., and Liddle, C. (1999) *Mol Pharmacol* **56**(6), 1329-39
19. Kocarek, T. A., Kraniak, J. M., and Reddy, A. B. (1998) *Mol Pharmacol* **54**, 474-84
20. Kocarek, T. A., and Reddy, A. B. (1998) *Biochem Pharmacol* **55**(9), 1435-43
21. Kocarek, T. A., Schuetz, E. G., and Guzelian, P. S. (1993) *Toxicol Appl Pharmacol* **120**(2), 298-307
22. Ourlin, J. C., Handschin, C., Kaufmann, M., and Meyer, U. A. (2002) *Biochem Biophys Res Commun* **291**(2), 378-84
23. Setchell, K. D., Rodrigues, C. M., Clerici, C., Solinas, A., Morelli, A., Gartung, C., and Boyer, J. (1997) *Gastroenterology* **112**(1), 226-35
24. Fischer, S., Beuers, U., Spengler, U., Zwiebel, F. M., and Koebe, H. G. (1996) *Clin Chim Acta* **251**(2), 173-86
25. Jones, S. A., Moore, L. B., Shenk, J. L., Wisely, G. B., Hamilton, G. A., McKee, D. D., Tomkinson, N. C., LeCluyse, E. L., Lambert, M. H., Willson, T. M., Kliewer, S. A., and Moore, J. T. (2000) *Mol Endocrinol* **14**(1), 27-39
26. Forman, B. M., Ruan, B., Chen, J., Schroepfer, G. J., Jr., and Evans, R. M. (1997) *Proc Natl Acad Sci U S A* **94**(20), 10588-93
27. Gan, X., Kaplan, R., Menke, J. G., MacNaul, K., Chen, Y., Sparrow, C. P., Zhou, G., Wright, S. D., and Cai, T. Q. (2001) *J Biol Chem* **276**(52), 48702-8
28. Mangelsdorf, D. J., and Evans, R. M. (1995) *Cell* **83**(6), 841-50
29. Araya, Z., and Wikvall, K. (1999) *Biochim Biophys Acta* **1438**(1), 47-54

30. Furster, C., and Wikvall, K. (1999) *Biochim Biophys Acta* **1437**(1), 46-52
31. Song, C., Hiipakka, R. A., and Liao, S. (2000) *Steroids* **65**(8), 423-7
32. Sueyoshi, T., Kawamoto, T., Zelko, I., Honkakoski, P., and Negishi, M. (1999) *J Biol Chem* **274**(10), 6043-6
33. Chawla, A., Repa, J. J., Evans, R. M., and Mangelsdorf, D. J. (2001) *Science* **294**(5548), 1866-70
34. Chiang, J. Y., Kimmel, R., and Stroup, D. (2001) *Gene* **262**(1-2), 257-65
35. Schuetz, E. G., Strom, S., Yasuda, K., Lecureur, V., Assem, M., Brimer, C., Lamba, J., Kim, R. B., Ramachandran, V., Komoroski, B. J., Venkataramanan, R., Cai, H., Sinal, C. J., Gonzalez, F. J., and Schuetz, J. D. (2001) *J Biol Chem* **276**(42), 39411-8.
36. Sugatani, J., Kojima, H., Ueda, A., Kakizaki, S., Yoshinari, K., Gong, Q. H., Owens, I. S., Negishi, M., and Sueyoshi, T. (2001) *Hepatology* **33**(5), 1232-8
37. Synold, T. W., Dussault, I., and Forman, B. M. (2001) *Nat Med* **7**(5), 584-90
38. Plewka, A., and Kaminski, M. (1996) *Exp Toxicol Pathol* **48**(4), 249-53
39. Watanabe, M., Nakura, H., Tateishi, T., Tanaka, H., Kumai, T., and Kobayashi, S. (1996) *Med Sci Res* **24**, 257-9
40. Andersson, M., Ericsson, J., Appelkvist, E. L., Schedin, S., Chojnacki, T., and Dallner, G. (1994) *Biochim Biophys Acta* **1214**(1), 79-87
41. Sudjana-Sugiaman, E., Eggertsen, G., and Bjorkhem, I. (1994) *J Lipid Res* **35**(2), 319-27
42. Blouin, R. A., Bandyopadhyay, A. M., Chaudhary, I., Robertson, L. W., Gemzik, B., and Parkinson, A. (1993) *Arch Biochem Biophys* **303**(2), 313-20
43. Thomas, K. D. (1984) *Isr J Med Sci* **20**(3), 240-1
44. Eiris, J. M., Lojo, S., Del Rio, M. C., Novo, I., Bravo, M., Pavon, P., and Castro-Gago, M. (1995) *Neurology* **45**(6), 1155-7
45. Aynaci, F. M., Orhan, F., Orem, A., Yildirmis, S., and Gedik, Y. (2001) *J Child Neurol* **16**(5), 367-9.

46. Schwaninger, M., Ringleb, P., Annecke, A., Winter, R., Kohl, B., Werle, E., Fiehn, W., Rieser, P. A., and Walter-Sack, I. (2000) *J Neurol* **247**(9), 687-90
47. Verrotti, A., Domizio, S., Angelozzi, B., Sabatino, G., Morgese, G., and Chiarelli, F. (1997) *J Paediatr Child Health* **33**(3), 242-5
48. Yilmaz, E., Dosan, Y., Gurgoze, M. K., and Gungor, S. (2001) *Acta Neurol Belg* **101**(4), 217-20
49. Penzak, S. R., and Chuck, S. K. (2000) *Scand J Infect Dis* **32**(2), 111-23
50. Dussault, I., Lin, M., Hollister, K., Wang, E. H., Synold, T. W., and Forman, B. M. (2001) *J Biol Chem* **276**(36), 33309-12
51. Back, P. (1987) *Pharmacol Ther* **33**(1), 153-5
52. Summerfield, J. A., Billing, B. H., and Shackleton, C. H. (1976) *Biochem J* **154**(2), 507-16
53. Wietholtz, H., Marschall, H. U., Sjoval, J., and Matern, S. (1996) *J Hepatol* **24**(6), 713-8
54. Cancado, E. L., Leitao, R. M., Carrilho, F. J., and Laudanna, A. A. (1998) *Am J Gastroenterol* **93**(9), 1510-7
55. Chawla, A., Saez, E., and Evans, R. M. (2000) *Cell* **103**(1), 1-4
56. Repa, J. J., Turley, S. D., Lobaccaro, J. A., Medina, J., Li, L., Lustig, K., Shan, B., Heyman, R. A., Dietschy, J. M., and Mangelsdorf, D. J. (2000) *Science* **289**(5484), 1524-9
57. Venkateswaran, A., Repa, J. J., Lobaccaro, J. M., Bronson, A., Mangelsdorf, D. J., and Edwards, P. A. (2000) *J Biol Chem* **275**(19), 14700-7
58. Sehayek, E., Ono, J. G., Duncan, E. M., Batta, A. K., Salen, G., Shefer, S., Neguyen, L. B., Yang, K., Lipkin, M., and Breslow, J. L. (2001) *J Lipid Res* **42**(8), 1250-6
59. Blumberg, B., and Evans, R. M. (1998) *Genes Dev* **12**(20), 3149-55

Figure Legends

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 hours in medium lacking serum. Cells were subsequently treated for 16 hours with either vehicle (0.1 % DMSO), 400 μ M PB, 10 μ M clotrimazole, 10 μ M 22(R)-hydroxycholesterol (22R), 10 μ M 24(S)-hydroxycholesterol (24S) or 20 μ M 25-hydroxycholesterol (25O) 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 μ M cholic acid (CA), 100 μ M deoxycholic acid (DCA), 100 μ M chenodeoxycholic acid (CCA) or 400 μ M 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 % DMSO), 400 μ M phenobarbital, 10 μ M clotrimazole, 10 μ M 20 α -hydroxycholesterol (20 α), 10 μ M 22(R)-hydroxycholesterol (22R), 10 μ M 24(S)-hydroxycholesterol (24S) or 20 μ M 25-hydroxycholesterol (25O) alone or in combinations for 24 hours. Cell extracts were analyzed for CAT expression normalized against β -galactosidase levels. **d**, Transfected CV-1 cells were treated with 100 μ M cholic acid (CA), 100 μ M deoxycholic acid (DCA), 100 μ M chenodeoxycholic acid (CCA) or 400 μ M PB. Values are the mean of three independent experiments, and bars represent standard deviation.

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 RXR γ (lanes 2 and 6-11) and anti-RXR antibody (lanes 9-11). Arrows depict the unbound probe (a), the complex of CXR and LXR with RXR γ (b) and the supershift of these complexes after addition of anti-RXR antibody (c). **b**, Radiolabeled wildtype 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 RXR γ (lanes 3-6). Arrows depict the unbound probe (a) and the complex of CXR and LXR with RXR γ (b). **c**, Radiolabeled wildtype 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 RXR γ (lanes 2-13) and anti-HA antibody (lanes 2-13) as indicated. Increasing HA-CXR concentrations were applied from lane 3 to lane 12. Arrows depict the unbound probe (a), the complex of HA-CXR with RXR γ (b), the shift of the LXR/RXR γ complex bound to the 264-bp PBRU (c) and the supershift of HA-CXR and RXR γ together with anti-HA antibody (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.

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 hours in medium without serum. Cells were subsequently treated for 16 hours with either vehicle (0.1 % DMSO), 400 μ M PB or increasing concentrations of 9-cis-retinoic acid (0.1 nM–10 μ M). Cells were harvested and CAT levels determined. The relative CAT expression was standardized against PB-treated cells and expressed in percentage of PB-induction. **b**, LMH transfected with the 264-bp PBRU were treated for 16 hours with vehicle, 400 μ M PB, 10 μ M 22(R)-hydroxycholesterol (22R), 0.1 μ M 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 standard deviation. **c**, Transfected LMH cells were treated with 400 μ M phenobarbital (PB) or 25 μ M of the LXR-inhibitor geranylgeranyl-pyrophosphate (GGPP) for 16 hours before CAT levels were measured.

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 hours in medium lacking serum. Cells were subsequently treated for 16 hours with either vehicle (0.1 % DMSO), 400 μ M PB, 10 μ M clotrimazole, 10 μ M 24(S)-hydroxycholesterol (24S), 10 μ M hyocholic acid (HC), 10 μ M hyodeoxycholic acid (HD) or combinations of these compounds. The relative CAT expression was standardized against untreated cells and expressed as fold induction. **b, c**, CV-1 cells were co-transfected with the GAL4(DBD)-LXR(LBD) (Fig. 4b) or the GAL4(DBD)-FXR(LBD) (Fig. 4c) fusion proteins, respectively, together with the reporter gene plasmid (UAS)5-tk-CAT. Cells were subsequently treated either with vehicle (0.1 % DMSO), 10 μ M 19-hydroxycholesterol (19O), 10 μ M 20 α -hydroxycholesterol (20 α), 10 μ M 24(S)-hydroxycholesterol (24S), 10 μ M 22(R)-hydroxycholesterol (22R), 20 μ M 25-hydroxycholesterol (25O), 10 μ M cholic acid (CA), 10 μ M deoxycholic acid (DCA) or 10 μ M chenodeoxycholic acid (CCA), 10 μ M hyocholic acid (HC), 10 μ M hyodeoxycholic acid (HD), 25 μ M geranylgeranyl pyrophosphate (GGPP) or combinations of these drugs for 24 hours. Cell extracts were analyzed for CAT expression normalized against β -galactosidase levels. Values represent the mean of three independent experiments, and bars represent standard deviation.

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 wildtype XREM of human CYP3A4 or 51-bp PBREM of CYP2B6 as well as the corresponding PBREM sequence containing a double mutation in the DR-4 context were incubated with in vitro transcribed/translated human PXR, CAR or LXR α together with RXR α . **b**, Amplification of nuclear receptors from cDNA of CV-1 cells. Specific primers against human CAR (lane 1), PXR (lane 2), LXR α (lane 3), LXR β (lane 4), FXR (lane 5) and of glyceraldehyde-3-phosphate dehydrogenase (GAPDH, lane 6) were used for PCR reactions using CV-1 cell cDNA as template. Regions of the expected sizes were excised, subcloned and sequenced to confirm the identity of the

bands as described in the Methods section. **c, d, 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% DMSO), 400 μ M phenobarbital, 10 μ M 25-hydroxycholesterol or combinations of these drugs for 24 hours, cell extracts were analyzed for CAT expression normalized against β -galactosidase levels. Values are the mean of three independent experiments, and bars represent standard deviation.

FIG 6. Chicken CYP7A1 expression is inhibited by bile acids and phenobarbital. Total RNA from LMH cells treated with vehicle, 400 μ M PB, 20 μ M 25-hydroxycholesterol (25-OHC) or 100 μ M CCA was isolated and analyzed on blots probed with radiolabeled chicken CYP7A1 or chicken GAPDH.

FIG 7. Proposed regulatory interplay between drug-metabolising 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.