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The Small Heterodimer Partner Interacts with the Pregnane X Receptor and Represses Its Transcriptional Activity

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SHP (small heterodimer partner, NR1I0) is an atypical orphan member of the nuclear receptor subfamily in that it lacks a DNA-binding domain. It is mostly expressed in the liver, where it binds to and inhibits the function of nuclear receptors. SHP is up-regulated by primary bile acids, through the activation of their receptor farnesoid X receptor, leading to the repression of cholesterol 7α-hydroxylase (CYP7α) expression, the rate-limiting enzyme in bile acid production from cholesterol. PXR (pregnane X receptor, NR1I2) is a broad-specificity sensor that recognizes a wide variety of synthetic drugs as well as endogenous compounds such as bile acid precursors. Upon activation, PXR induces CYP3A and inhibits CYP7α, suggesting that PXR can act on both bile acid synthesis and elimination. Indeed, CYP7α and CYP3A are involved in biochemical pathways leading to cholesterol conversion into primary bile acids, whereas CYP3A is also involved in the detoxification of toxic secondary bile acid derivatives. Here, we show that PXR is a target for SHP. Using pull-down assays, we show that SHP interacts with both murine and human PXR in a ligand-dependent manner. From transient transfection assays, SHP is shown to be a potent repressor of PXR transactivation. Furthermore, we report that chenodeoxycholic acid and cholic acid, two farnesoid X receptor ligands, induce up-regulation of SHP and provoke a repression of PXR-mediated CYP3A induction in human hepatocytes as well as *in vivo* in mice. These results reveal an elaborate regulatory cascade, tightly controlled by SHP, for both the maintenance of bile acid production and detoxification in the liver. (*Molecular Endocrinology* 17: 1693–1703, 2003)

NUCLEAR RECEPTORS CONSTITUTE a superfamily of ligand-modulated transcription factors that mediate cellular response to small lipophilic endogenous and exogenous ligands. They consist of a variable N-terminal domain, often exhibiting a constitutive transcription activation function (AF-1), a highly conserved zinc finger type DNA-binding domain (DBD), a variable linker region, and a multifunctional C-terminal domain that is responsible for ligand binding (LBD), dimerization, and ligand-induced transcriptional AF-2 (1). Most nuclear receptors heterodimerize with the retinoid X receptor (RXR).

SHP (short heterodimer partner) is an orphan nuclear receptor that lacks a conventional DBD and is mostly expressed in the liver (2). In spite of the lack of a DBD or of a conventional nuclear localization signal, it appears to be located in the nucleus of mammalian transfected cells (3). A series of reports have shown that SHP is able to interact with and inhibit the transcriptional activity of several members of the nuclear receptor superfamily including the constitutive androstane receptor (CAR), thyroid hormone receptor (TR), retinoid X receptor (RXR), retinoid acid receptor (RAR), androgen receptor, estrogen receptor (ER), the liver receptor homolog-1 (LRH-1), liver X receptor α and β, and hepatocyte nuclear factor-4 (HNF4) (3–11). SHP has been shown to inhibit RAR:RXR heterodimer DNA binding and to inhibit the transcriptional activity of RAR, mCAR, and ER. Moreover, as SHP contains a strong transcriptional repression domain in its C terminus (3), it has been suspected to act as a direct transcriptional repressor by recruiting conventional corepressors such as nuclear receptor corepressor, as

Abbreviations: AF, Activation function; CA, cholic acid; CAR, constitutive androstane receptor; CDCA, chenodeoxycholic acid; CYP7α, cholesterol 7α-hydroxylase; DBD, DNA-binding domain; DMSO, dimtheylsulfoxide; FXR, farnesoid X receptor; GAL4, positive regulator of galactose inducible genes in yeast; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GST, glutathione-*S*-transferase; HNF4, hepatocyte nuclear factor-4; h, human; LRH-1, liver receptor homolog-1; m, mouse; mSHP, murine SHP fusion protein; PCN, pregnenolone 16-carbonitrile; PXR, pregnane X receptor; RAR, retinoic acid receptor; RIF, rifampicin; RXR, retinoid X receptor; SHP, small heterodimer partner; SRC-1, steroid receptor coactivator-1; TR, thyroid hormone receptor; UT, untreated; XREM, xenobiotic-responsive element module.

reported with DAX-1 (DSS-AHC critical region on X chromosome, gene 1) (12). However, further analysis failed to demonstrate a direct interaction between SHP and nuclear receptor corepressor (3). Alternatively, SHP has recently been proposed to represent a new category of nuclear receptor coregulator, interfering directly with AF-1 and AF-2 coactivator factors such as coactivator four-and-a-half-LIM-only protein FHL2, steroid receptor coactivator-1 (SRC-1), transcription intermediary factor 2, or receptor interacting protein with a molecular mass of 140 kDa, and two possibilities have been considered in this respect: 1) SHP and AF-1/2 coactivators may compete for a common site; or 2) binding of SHP to the receptor may induce conformational changes leading to the dissociation of AF-1/2 coactivators from the receptors (3, 5, 13). Taken together, these studies suggest that SHP inhibits the transcriptional activity of nuclear receptors by several mechanisms.

Recently, SHP has been shown to be involved in the control of bile acid biosynthesis, the first and rate-limiting step of which is catalyzed by cytochrome P450 7α (CYP7α), a liver-specific enzyme (14). Two different laboratories (4, 9) have reported that upon activation by primary bile acids such as chenodeoxycholic acid (CDCA), farnesoid X receptor (FXR) induces the expression of SHP, which then binds to and inhibits LRH-1, an orphan receptor that regulates CYP7α expression (15).

CYP3A enzymes are known to be involved in the oxidative metabolism of many xenobiotics as well as of endogenous compounds such as steroids (16). More recently, these enzymes have been shown to play a role in bile acid catabolism and biosynthesis (17, 18). Fuster and Wikvall (18) demonstrated that microsomal 25-hydroxylation of 5β-cholestane-3α,7α,12α-triol, a known bile acid precursor, is catalyzed mainly by CYP3A4. In addition, Honda *et al.* (19) demonstrated that microsomal 25- and 26-hydroxylation of the cholesterol side chain are catalyzed by cyp3a11 in CYP27$-/-$ mice, and they provided strong arguments in favor of the implication of human CYP3A4 in a similar function. Notably, using human liver microsomes, they reported that the rates of 5β-cholestane-3α,7α,12α-triol 25- and 26-hydroxylation and 5β-cholestane-3α,7α,12α, 25-tetrol 23R-,24R-, 24S- and 27-hydroxylation are strongly correlated with the rate of 6β-hydroxylation of testosterone, a marker of CYP3A4. Finally, Cheng *et al.* (20) reported in 1977 that phenobarbital, a well-known CYP3A inducer, produces an increase in the rate of hydroxylation of 5β-cholestane-3α,7α,12α-triol and 5β-cholestane-3α,7α,12α,25-tetrol in rats. These metabolite products can be either excreted from the body in the bile or urine or converted to cholic acid (CA).

PXR (pregnane X receptor) was first reported to control CYP3A gene induction upon activation by xenobiotic inducers such as phenobarbital and rifampicin (RIF) (21). More recently, it has been shown that PXR is a functional receptor of the bile acid precursors (5β-cholestan-3α-7α-12α-triol; and 5β-cholestan-3α-7α-12α-25-tetrol) (22, 23), which represent CYP3A substrate and product, respectively. PXR is also activated by secondary bile acid derivatives such as lithocholic acid (24). Upon activation, PXR controls elimination of these compounds by inducing CYP3A4 (21) and Oatp2 (24, 25) expression. However, the concentration of lithocholic acid required to activate PXR appears to be higher than those that occurs *in vivo*.

A Series of Recent Reports Suggest that CYP3A Expression Is Negatively Regulated by FXR Ligands and/or FXR

1) Taurochenodeoxycholic acid, a FXR ligand (26), reduced CYP3A-associated monooxygenase activities *in vivo* in rats (27). 2) Cyp3a11 up-regulation observed in CYP27 $-/-$ mice is reversed if mice are fed with diets containing CA or CDCA (22). 3) Handschin *et al.* (28) reported that cotreatments with CA or CDCA and phenobarbital or clotrimazole (activators of CAR and PXR, respectively) reduces CYP3A induction in a chicken hepatoma cell line LMH. 4) Finally, Schuetz *et al.* (29) observed a strong increase in the expression of cyp3a11 and cyp2b10 in FXR $-/-$ mice with respect to wild-type animals. However, the molecular mechanisms involved remain largely unknown. As FXR has been shown not to bind to PXR- or CAR-responsive elements (28), we suspected that these negative effects on CYP3A expression could be mediated through FXR-induced SHP up-regulation. Experiments reported here, using both *in vitro* and *in vivo* experiments, show that the transcriptional activity of the ligand-activated form of PXR is repressed by SHP.

RESULTS

PXR Interacts with SHP in a Ligand-Dependent Manner

Interaction between SHP and PXR was investigated biochemically *in vitro*, using the glutathione-*S*-transferase (GST) pull-down assay. As indicated in Fig. 1A, ^{35}S-methionine-labeled murine PXR.1 interacted with the GST-murine SHP fusion protein (mSHP) in the presence of pregnenolone 16-carbonitrile (PCN) in a dose-dependent manner (lanes 5 and 6), whereas a very faint interaction was observed in the absence of PCN (lane 4). No interaction was observed with the control GST protein (lane 3). As expected, a ligand-independent interaction was observed between GST-mSHP and mCAR (lane 2), but no interaction was observed with radiolabeled luciferase protein (lane 1). A similar interaction was observed with an opposite approach between the GST-human (h) PXR fusion protein and ^{35}S-methionine-labeled mSHP in the presence of RIF (Fig. 1B, lanes 2 and 3). As expected, luciferase protein failed to interact with the GST-hPXR fusion protein (lane 5), whereas no interaction was

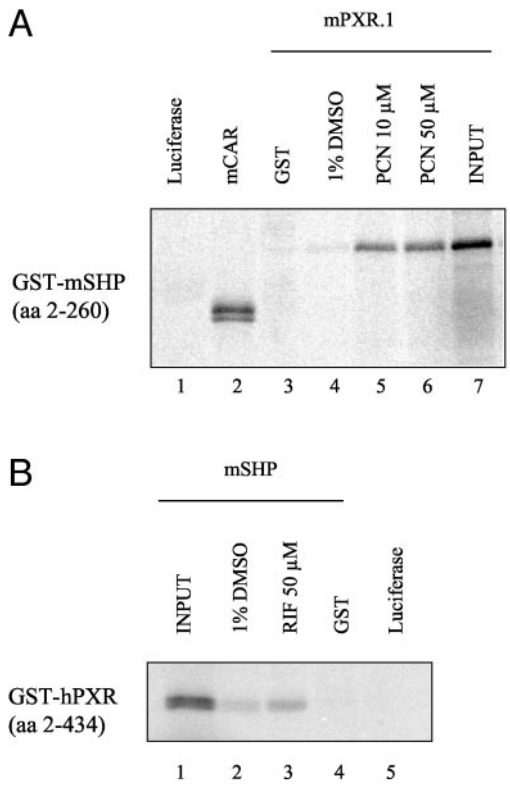

Fig. 1. *In Vitro* Interaction between Ligand-Activated PXR and SHP in GST-Fusion Protein Based Assays

A, *In vitro* interaction between GST-mSHP fusion protein and mPXR.1. Murine PXR.1, CAR, or luciferase proteins were labeled with ^{35}S-methionine by *in vitro* translation. GST or GST-murine SHP fusion protein were expressed in *E. coli* BL21 strain and tested for interaction with mPXR.1 in the absence and presence of increasing amount of PCN. B, *In vitro* interaction between GST-human PXR fusion protein and SHP. Murine SHP or luciferase proteins were labeled with ^{35}S-methionine by *in vitro* translation. GST or GST-human PXR fusion protein were expressed in *E. coli* BL21 strain and tested for interaction with SHP in the absence and presence of RIF. About 20% of mSHP was used in each lane are shown as INPUT.

observed with the control GST protein (lane 4). Overall, these *in vitro* assays demonstrate that SHP interacts with PXR in a ligand-enhanced manner and suggest that PXR could be a target for SHP-mediated repression.

SHP Inhibits the Transcriptional Activity of PXR

SHP has previously been shown to act as a negative regulator of several receptor signaling pathways in mammalian cells (2). The effect of SHP on the transcriptional activity of mouse (m) PXR and hPXR was investigated by transient cotransfections using a reporter plasmid containing only three copies of the PXR response element of the mouse CYP3A promoter in front of the minimal thymidine kinase promoter (30), *i.e.* (CYP3A1 DR3)3-tkCAT, or the previously described reporter construct containing the proximal CYP3A4 promoter (−1100/+43) linked to the distal xenobiotic-responsive element module (XREM) of the human CYP3A4 gene (31, 32) *i.e.* p(CYP3A4 XREM-[−7800/+7200]/−1100/+43)-pGL3-LUC. These reporter plasmids were cotransfected with mPXR or hPXR expression plasmids and increasing amounts of mSHP or hSHP expression plasmids, respectively. The results obtained in CV1 cells are shown in Fig. 2. In these experiments, PCN and RIF were used as specific ligands of mPXR and hPXR, respectively. It clearly appears that cotransfection of increasing amounts of mSHP or hSHP expression plasmids resulted in a dose-dependent decrease in the ligand-induced transactivation of both reporter constructs by mPXR (Fig. 2A) or hPXR (Fig. 2B), with only approximately 20% of residual activity in the presence of the higher amount of SHP expression plasmids. This level of inhibition is close to that previously reported with mCAR and RXR, and stronger than that observed with RAR (2). Similar results were obtained in HepG2 cells (data not shown) so that SHP is able to interact with hPXR in two different cell lines. Consistent with the relatively weak ligand-independent interaction of SHP with PXR observed *in vitro* (Fig. 1), SHP coexpression resulted in a moderate decreased of PXR transcriptional activity in the absence of ligand (20–40% of inhibition). This inhibition may result from different mechanisms including: 1) SHP *vs.* RXR competitive binding to PXR leading to impairment of PXR:RXR heterodimer binding to DNA as suggested for RAR:RXR (10); or 2) SHP-PXR interaction involving the repression domain and/or the LBD/AF-2 domain of PXR as reported for the ER (3).

SHP Inhibits PXR:RXR Heterodimer DNA Binding

Although its closest relative receptor, DAX-1, is able to bind to the retinoid acid response element of the RAR element β2 promoter (33), SHP is not (3, 10). Using gel mobility shift assays and *in vitro* synthesized proteins, we confirmed that SHP alone or in the presence of either RXR or PXR does not bind to the CYP3A4 ER6 oligonucleotide (not shown), whereas, as expected, the PXR:RXRα heterodimer does (Fig. 3, lane 4). We then verified that this complex is displaced by an excess of ER6 unlabeled oligonucleotide (lane 5). Interestingly, bacteria-expressed GST-SHP inhibited the binding of PXR:RXR heterodimer to the ER6 in a dose-dependent manner (lanes 8–9), whereas comparable amounts of GST did not (lanes 6–7). It is tempting to propose from these results that the inhibitory effect of SHP on PXR transcriptional activity results from the formation of a SHP:PXR heterodimer that is unable to bind the PXR-responsive element. However, as SHP is able to interact with RXR in the absence of ligand, this inhibitory effect could merely result from a depletion of RXR through the formation of SHP:RXR heterodimers.

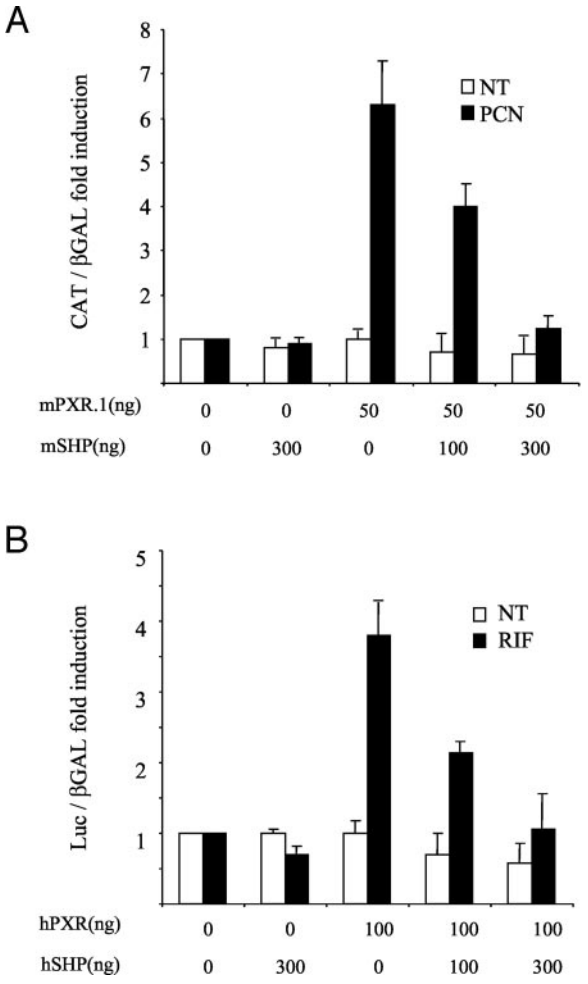

Fig. 2. SHP Inhibits PXR-Dependent Transactivation in Transient Transfection Assays

A, CV1 cells were cotransfected with 50 ng of mPXR.1 expression plasmid and increasing amounts of mSHP expression plasmid in combination with 250 ng of the (CYP3A DR3)3 tk-CAT reporter and pSV-β-galactosidase (25 ng) as internal control. Cells were then incubated 24 h with solvent (NT) or 5 μM PCN, and assayed for CAT and β-galactosidase activities. B, CV1 cells were cotransfected with 100 ng of pSG5-Δ^{ATG}-hPXR expression plasmid and increasing amount of hSHP expression plasmid in combination with 250 ng of p(CYP3A4/XREM/−1100/+43)-tk-LUC reporter and pSV-β-galactosidase (25 ng) as internal control, as indicated. Cells were then incubated for 24 h with solvent (NT) or 5 μM RIF, and assayed for luciferase and β-galactosidase activities. All values represent the mean of duplicate samples, and are derived from a representative experiment among three independent ones.

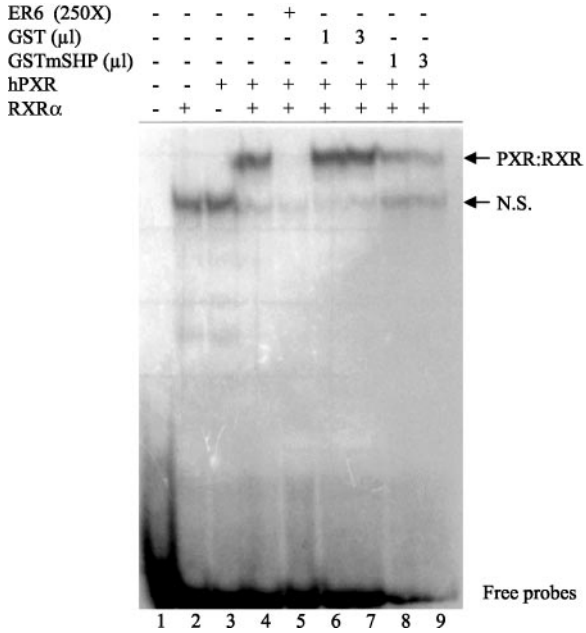

Fig. 3. SHP Inhibits PXR:RXR Heterodimer Binding to CYP3A4 ER6 Oligonucleotide

EMSAs were performed with a ^{32}P-labeled oligonucleotide containing the ER6 PXR binding site from the human CYP3A4 promoter and *in vitro* synthetized Δ^{ATG}-hPXR and RXRα in combination with increasing amounts of purified GST-mSHP or GST. The positions of the shifted PXR: RXR: ER6 complex (PXR:RXR), nonspecific band (N.S.) and free probes are indicated.

SHP Inhibits the Transcriptional Activity of a Positive Regulator of Galactose Inducible Genes in Yeast (GAL4)-hPXR Ligand Binding Domain Fusion Protein

In an attempt to determine whether the inhibitory effect of SHP involves the LBD of PXR, we used a fusion protein consisting of the GAL4-DNA binding domain linked to the LBD/AF-2 domain of hPXR (encoded by plasmid pM-LBDhPXR). pM-LBDhPXR was cotransfected in HepG2 cells in the presence of a GAL4-responsive luciferase reporter gene (plasmid 17mx5-Glob-LUC) with or without increasing amounts of mSHP or hSHP expression plasmids. Reporter expression assay showed that RIF-enhanced luciferase expression only in the presence of pM-LBDhPXR (Fig. 4). The cotransfection of both mSHP or hSHP expression vectors resulted 1) in a moderate but dose-dependent reduction of pM-LBDhPXR basal transactivation of the reporter, and 2) a stronger decrease of the RIF-induced enhancement of luciferase expression. In addition, inhibitions of LBD-hPXR by hSHP appeared somewhat stronger that those observed with mPXR. These results show that the inhibitory effect of SHP on PXR might be mediated through the LBD/AF-2 domain of PXR.

The AF-2 Coactivator SRC-1 Antagonizes the Inhibitory Effect of SHP on PXR in Mammalian Cells

Recently, it has been proposed that SHP inhibits the ER transcriptional activity by acting as a negative AF-2 coregulator (3, 6, 13). In fact, whereas SHP binds to the ligand activated ER, it does not inhibit ER homodimer DNA binding. GST pull-down and mamma-

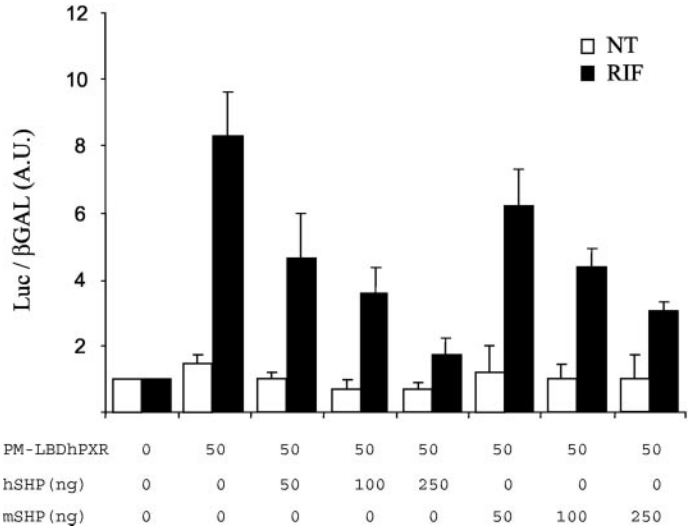

Fig. 4. mSHP and hSHP Inhibit GAL4-LBDhPXR-Dependent Transactivation in Transient Transfection Assays

HepG2 cells were cotransfected with 50 ng pM-LBD hPXR with or without increasing amount of mSHP or hSHP expression plasmids (50–250 ng) in combination with 200 ng of the 17mx5-βGlob-LUC containing five GAL4 binding sites upstream of the luciferase reporter gene. pSV-β-galactosidase (25 ng) was added as internal control, and pM empty vector was added to keep constant the total amount of DNA transfected. Cells were then incubated for 24 h with solvent (NT) or 5 μM RIF, and assayed for LUC and β-galactosidase activities.

lian cell cotransfection experiments revealed that SHP competes with AF-2 coactivators (transcription intermediary factor 2 and receptor-interacting protein with a molecular mass of 140 kDa) for binding to the ligand-activated ER. We therefore decided to test whether such interference between SHP and coactivators could explain our observations on PXR. SRC-1 is a member of the P160/SRC-1 family of positive AF-2 coactivators that bind to the LBD/AF-2 region of ligand-activated nuclear receptors and enhance their transcriptional activity (34). Notably, SRC-1 interacts with PXR (21). Using cotransfection assays in CV1 cells, we first verified that SRC-1 significantly enhances PXR transcriptional activity (Fig. 5). More interestingly, whereas SHP inhibits PXR-mediated reporter gene expression, the cotransfection of increasing amounts of SRC-1 expression plasmid partially abrogates the inhibitory effect of SHP on the transcriptional activity of PXR and suggest that SHP may act as a negative AF-2 coregulator of PXR.

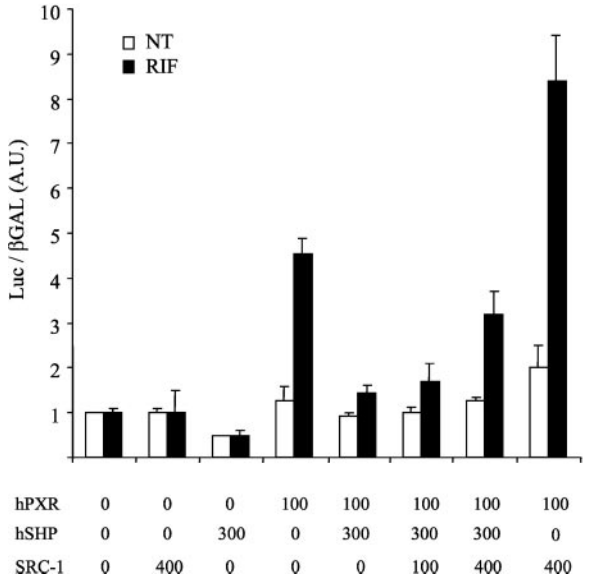

Fig. 5. SHP-Mediated Inhibition of Transcriptional Activity of PXR Is Reversed by SRC-1

HepG2 cells were cotransfected with the p(CYP3A4/XREM/−1100/+43)-tk-LUC reporter plasmid (250 ng) together with different combinations of expression plasmids for pSG5-Δ^{ATG}-hPXR, hSHP, and mSRC-1 as indicated with pSV-β-galactosidase (25 ng) as internal control. Cells were then incubated for 24 h with solvent (NT) or 5 μM RIF, and assayed for luciferase and β-galactosidase activities.

Bile Acid-Induced SHP Expression Correlates with the Inhibition of PXR Function in Human Hepatocytes and in Mice

The data presented above collectively show that SHP acts as a repressor of PXR function. As SHP gene expression is regulated by bile acid-activated FXR (4, 9), we next decided to examine the effect of FXR ligands such as CA and CDCA (26), on the PXR-mediated induction of CYP3A *in vitro* in human hepatocytes (Fig. 6) and *in vivo* in mice (Fig. 7). For this purpose, human hepatocytes were first pretreated for 72 h with increasing concentrations of CDCA (10–50 μM) and then treated for 16 h with 5 μM RIF. The level of CYP3A4 and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) mRNA was examined at the same time by Northern blotting analysis. As expected, RIF

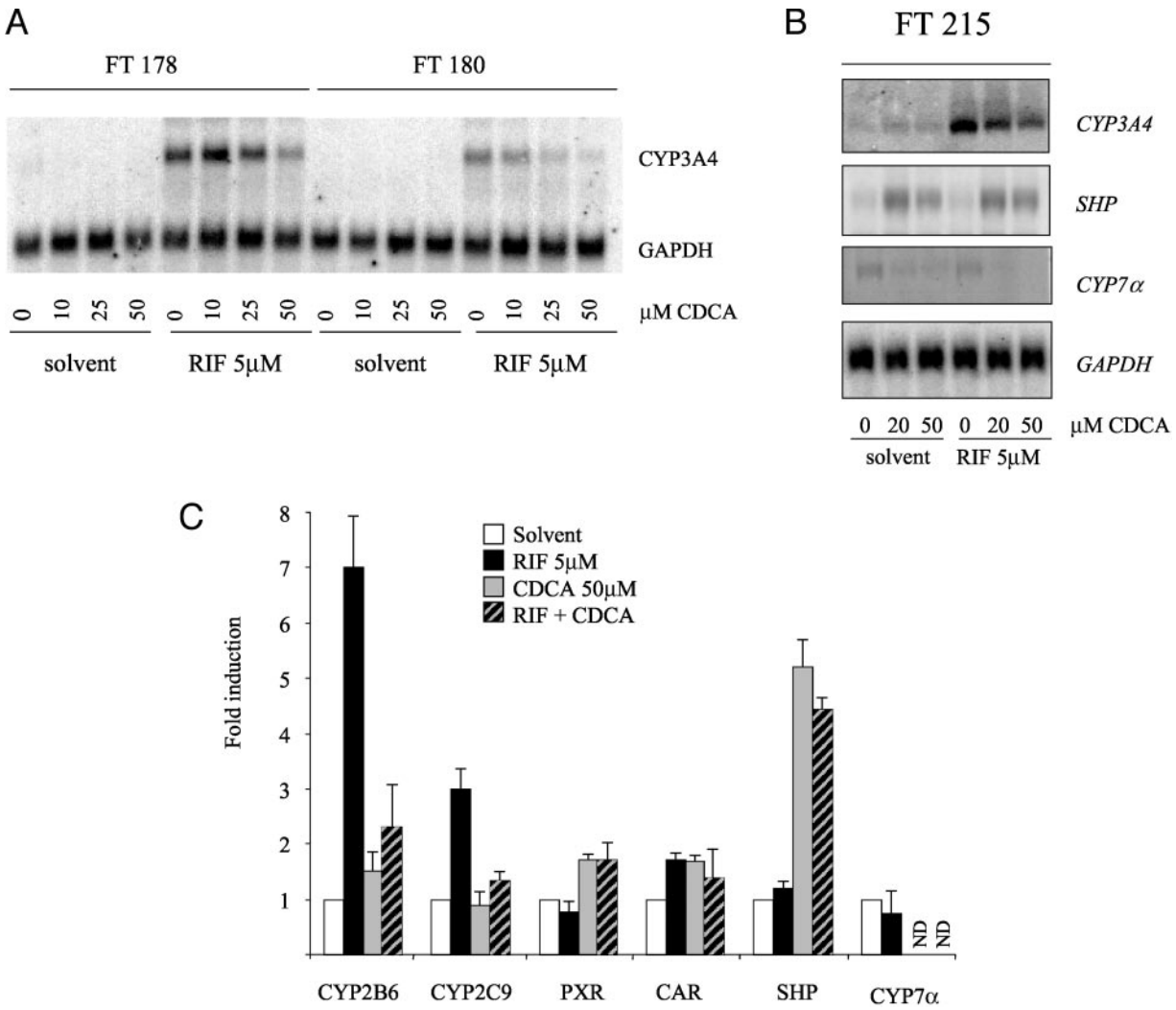

Fig. 6. The FXR Ligand CDCA Represses RIF-Mediated PXR Target Genes Expression in Human Hepatocytes

A, Primary human hepatocytes (FT 178 and FT 180) were pretreated 72 h with increasing concentrations of CDCA (10, 25, or 50 μM) and then cultured for 16 h with or without 5 μM RIF. Northern blot analysis was performed onto 20 μg of total RNA using specific CYP3A4 and GAPDH probes. B, Human hepatocytes (FT 215) were pretreated 72 h with increasing concentrations of CDCA (20 or 50 μM) and then cultured for 16 h with or without 5 μM RIF. Northern blot analysis was performed on 20 μg of total RNA using specific CYP3A4, GAPDH, SHP, and CYP7α probes. C, Primary human hepatocytes (FT 178, FT 180, and FT 215) were pretreated 72 h with 50 μM CDCA and then cultured for 16 h with or without 5 μM RIF. cDNAs were prepared from 1 μg of total RNA using reverse transcriptase and a tenth of this was used to determined the levels of CYP2B6, CYP2C9, PXR, CAR, CYP7α, and GAPDH mRNAs by real time RT-PCR analysis using the Light Cycler apparatus (Roche). Data presented are means of the ratio of mRNA levels compared with untreated cells, normalized with respect to GAPDH mRNA levels. ND, Undetectable.

provoked an induction of CYP3A4, whereas CDCA had no effect (Fig. 6, A and B). However, pretreatment of cells with CDCA resulted in a concentration-dependent inhibition of RIF-mediated induction of CYP3A4 mRNA, so that, in the presence of 50 μM CDCA, CYP3A4 mRNA expression was reduced by approximately 70% (n = 3, FT178, FT180, and FT215). As expected, CDCA treatment alone provoked an increase in SHP mRNA expression (5-fold induction) and a total inhibition of CYP7α mRNA expression as shown in Fig. 6, B and C. RIF-mediated CYP2B6 and CYP2C9 expressions were also decreased by CDCA pretreatment as shown in Fig. 6C, whereas PXR and CAR expression was not decrease by CDCA treatment. These results, obtained in a fully functional and highly differentiated cellular system, confirm that activation of SHP expression by a bile acid coincides with a strong reduction of PXR-mediated CYP expression. In addition, in CV1 cells cotransfected with the pM-LBDhPXR and a GAL4-responsive luciferase reporter gene, we observed no effect of CDCA nor CA on RIF-mediated luciferase expression (data not shown). These results rule out a possible antagonistic effect of these compounds on PXR.

To determine whether this process occurs *in vivo*, mice (n = 3) were fed a normal diet or a 1% CA-supple-

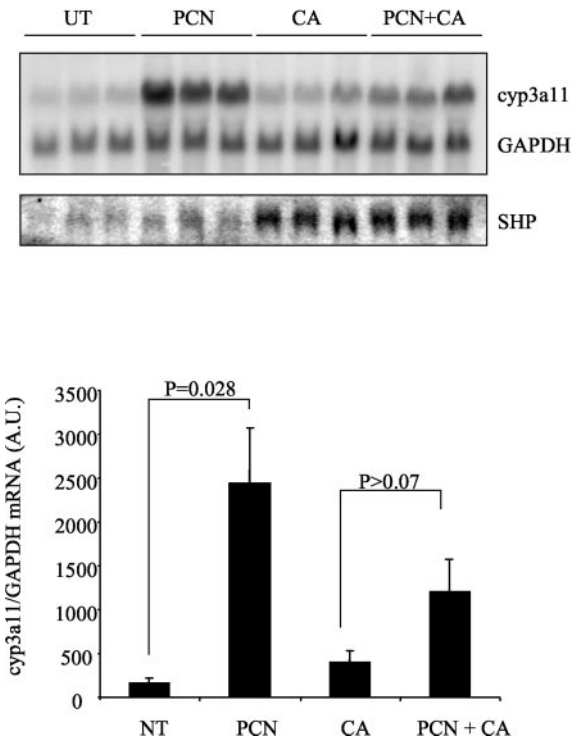

Fig. 7. CA Represses PXR-Mediated cyp3a11 Expression in Mice

Mice (n = 3) were fed normal chow or chow diets containing 1% CA (wt/wt) for 5 d and PCN (40 mg/kg) was injected in corn oil the last 2 d. Total RNA was isolated from individual livers. Northern blotting analysis were performed onto 30 μg of total RNA using CYP3A, GAPDH, and mSHP probes, quantified by PhosphorImager, and standardized against GAPDH and the results are shown as histogram. Statistically significant relative expressions were determined by Student's *t* test and *P* values are indicated.

mented diet for 3 d and were then treated for 2 d with PCN (40 mg/kg) or corn oil [untreated (UT)]. Total liver RNA was prepared and analyzed by Northern blotting with the indicated probes (CYP3A and GAPDH were analyzed at the same time, and then the membrane first stripped and then probed with radiolabeled mSHP cDNA). As shown in Fig. 7, cyp3a11 expression was strongly increased (≈14-fold) by PCN. More interestingly, CA pretreatment significantly decreased PCN-mediated cyp3a11 expression (50% inhibition compared with control mice treated with PCN), whereas it moderately induced cyp3a11 expression (≈2-fold). As expected, SHP expression was strongly increased by CA pretreatment. Thus, although there is a 14-fold induction of cyp3a11 gene expression by PCN in mice fed a normal diet, this value falls to approximately 3- to 4-fold induction in mice fed a 1% CA-supplemented diet.

DISCUSSION

Originally, SHP had been reported to bind to and inhibit CAR (2), a nuclear receptor involved in the induction of the CYP2 and CYP3 genes involved in the metabolism of xenobiotics in response to the prototypical inducer phenobarbital (35). Like CAR, PXR is a nuclear receptor that activates the CYP2 and CYP3A families in response to diverse chemicals and endogenous molecules. In addition, PXR regulates the expression of genes involved in the biosynthesis, transport, and metabolism of bile acids including CYP7α (36), Oatp2 (25), and CYP3A (21). Thus, it has been proposed that PXR serves as a physiological sensor not only for xenobiotics but also for bile acid precursors (22, 23). Our results suggest that SHP is a negative regulator of PXR transcriptional activity. This conclusion derives from *in vitro*, cell culture, and *in vivo* experiments.

GST pull-down assays demonstrated a direct interaction between SHP and both murine and hPXR which is enhanced by the presence of PXR ligands such as PCN and RIF, respectively. This interaction is specific as no binding was observed with our internal negative controls (GST alone or luciferase). Transient transfections in different cell lines and with different reporter constructs (*i.e.* native CYP3A4 promoter or repetitions of the minimal PXR responsive element), demonstrated that expression of SHP produced a dose-dependent inhibition of both murine and hPXR transcriptional activity and that this inhibition was reversed by the expression of SRC-1. This reversibility argues in favor of a functional and specific effect of SHP on PXR, and improves the understanding of the mechanism by which inhibition proceeds. Two complementary mechanisms can thus be proposed: 1) SHP interacts directly with PXR and weakens its binding to DNA as proposed previously for RAR; and 2) SHP blocks the AF-2 activation domain as proposed previously for ER (6).

We speculated that an overexpression of SHP would lead to a decrease in PXR activity and eventually to a decrease in CYP3A inducibility. SHP expression is under the control of FXR, which is activated by primary bile acids such as CA and CDCA (26). We have developed primary cultures of human hepatocytes in which many of the regulatory proteins (including aryl hydrocarbon receptor, CAR, PXR, glucocorticoid receptor, FXR, SHP) are expressed, whereas xenobiotic metabolism and hormonal regulation are fully maintained. As a consequence, the CYPs and others drug metabolizing enzymes are expressed and inducible in these cultures to an extent that is close to the *in vivo* situation (37, 38). The pretreatment of human hepatocytes with these compounds resulted in a clear overexpression of SHP. This overexpression was confirmed by the concomitant down-regulation of CYP7α, the rate-limiting enzyme in bile acid biosynthesis. Indeed, CYP7α is known to be subject to primary bile acid feedback regulation via SHP (4). In agreement with our hypothesis, PXR activation of CYP3A4, CYP2B6 and CYP2C9 genes was inhibited by FXR activators in a dose-dependent manner. These effects cannot result from a toxicity due to CDCA treatment

because both GAPDH and PXR mRNA expression were unaffected, and SHP mRNA level was up-regulated. Moreover, no detectable toxicity on cell culture has been noticed macroscopically (by examination under a microscope) or in terms of total RNA recovery. We finally used mice in an attempt to validate *in vivo* the observations made *in vitro* and in cellular models. Indeed, mice fed a CA-enriched diet exhibited a drastic decrease in PCN-induced cyp3a11 expression, whereas SHP was up-regulated.

The conversion of cholesterol to bile acids occurs exclusively in the liver. This process involves the translocation of cholesterol and intermediates through various compartments of the cell, where they encounter a wide variety of enzymes (CYP27A, CYP7α, CYP8B, CYP3A, etc.) necessary for their ultimate conversion to the primary C24 bile acids, CDCA and CA. Many nuclear receptors (liver X receptor, FXR, PXR) control this process through a complex network in which substrates and end-products modulate the up- and down-regulation of specific enzymes. According to our results, PXR function is inhibited in the presence of CDCA or CA through the up-regulation of SHP. This is reminiscent of the SHP-mediated repression of LRH-1 induced CYP7α expression. CYP7α, and to a lower extent CYP3A, promote cholesterol conversion into bile acids. The reason why primary bile acids repress PXR activity and, by consequence, the expression of those genes (including CYP3A) involved in the clearance of bile acids is still unclear. As CA and CDCA are nontoxic bile acid derivatives, in contrast to some bile acids precursors or secondary bile acids products, one explanation is that this process could prevent the over conversion of cholesterol into such toxic compounds through a regulatory feedback loop similar to that observed with CYP7α (Fig. 8).

The current findings are in agreement with previous observations. Handschin *et al.* (28) reported that cotreatment with CA or CDCA and phenobarbital or clotrimazole (activators of CAR and PXR, respectively) reduced CYP3A induction in a chicken hepatoma cell line LMH. In addition, Paolini *et al.* (27) reported that taurochenodeoxycholic acid (a FXR ligand) reduced CYP3A-associated monooxygenase activities *in vivo* in rats. Moreover, Schuetz *et al.* (29) observed a strong induction of cyp3a11 and cyp2b10 in FXR $-/-$ mice. Furthermore, in CYP27 $-/-$ mice, where CA and CDCA biosynthesis are abolished, there is an overaccumulation of cyp3a11 and CYP7α compared with wild-type mice (19, 22), whereas feeding of these mice diets containing CA or CDCA lead to up-regulation of SHP and down-regulation of Cyp3a11 or CYP7α (22).

In conclusion, we have shown that SHP interacts with and inhibits the transcriptional activity of PXR. As PXR controls the inducible expression of CYPs and other genes involved not only in the metabolism and elimination of xenobiotics but also in the biosynthesis and catabolism of bile acids, the current results reveal a functional interaction between bile acid homeostasis and the xenobiotic-mediated CYP induction in which SHP appears to play a major role.

MATERIALS AND METHODS

Drugs and Materials

DMEM culture medium, CA, CDCA, dimethylsulfoxide (DMSO), pregnenolone 16α-carbonitrile (PCN), RIF, and culture medium additives were from Sigma (Saint Louis, MO). γ-(^{32}P)deoxy (d)-ATP and α-(^{32}P)d-CTP were from Amersham International (Amersham, Buckinghamshire, UK).

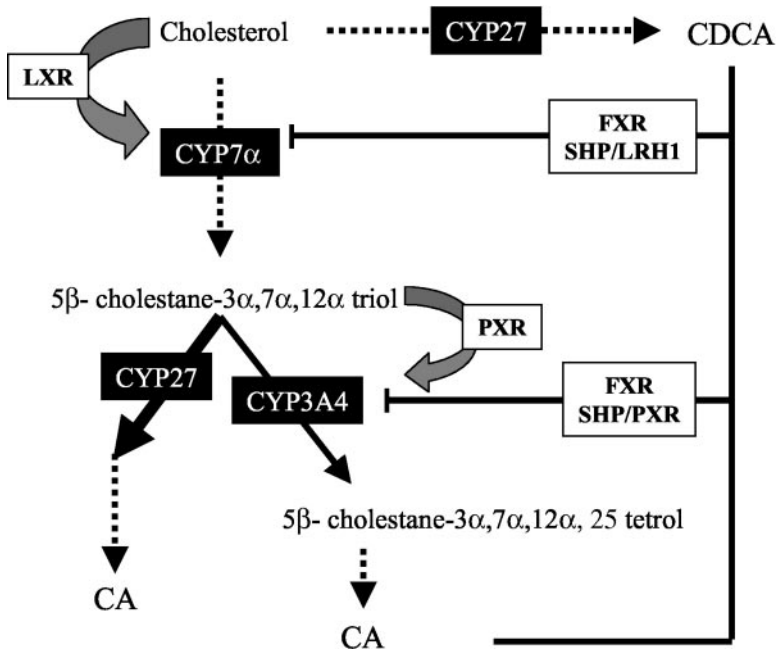

Fig. 8. Model Describing Feedback Regulations of Cholesterol Conversion into Bile Acids by Nuclear Receptors

Plasmids

The following plasmids have been described previously: pCR3-mCAR (39), pSG5-Δ^{ATG}-hPXR, p(CYP3A4 XREM-[−7800/+7200] −1100/+43)-pGL3-LUC (32), pSG5-mPXR.1, pSG5-hPXR and p(CYP3A1 DR3)3-tkCAT (21), CDM8-mSHP (2). pSG5-SRC-1 and GST-SRC-1 (amino acids 580–750) are from V. Cavailles, (Institut National de la Santé et de la Recherche Médicale, Montpellier, France). The pCMV-hSHP plasmid was given by Jun Takeda (40). The 17mx5-βGlob-LUC containing five GAL4 binding sites upstream of the luciferase reporter gene is from P. Chambon (Institut de Génétique Moléculaire et de Biologie Cellulaire, Strasbourg, France). The pM-LBDhPXR expression vector was generated by inserting a PCR fragment corresponding to the +107/STOP amino acids of the hPXR ligand binding domain (LBD) in the pM vector (CLONTECH). GST-mSHP fusion construct was generated by cloning the *Sma*I/*Xho*I fragment of the CDM8-mSHP into the EcoIRC1/*Xho*I sites of the pGEX-4T (Amersham). GST-hPXR fusion construct was generated by inserting the PCR-generated hPXR (amino acids 1–431) using oligonucleotides 5′-CCTCAGCTACCTGTGATGCCG and 5′-GGGTGTGGGGAATTCACCACCATGGAGGTG AGACCCAAAGAAAGC primers into pGEX-4T digested by *Eco*IRC1.

Cell Culture and Transfections

CV1 cells (Monkey kidney) or HepG2 cells (human hepatocarcinoma) were obtained from the American Type Culture Collection (Manassas, VA) and maintained in DMEM supplemented with 10% fetal calf serum, 100 mg/liter penicillin, and 100 mg/liter streptomycin (Life Technologies, Inc., Gaithersburg, MD). Transfection of plasmid DNA was performed in single batches with Fugene-6 (Roche Applied Science, Indianapolis, IN) as instructed by the manufacturer. Transfection was performed using 80,000 cells, and cell extracts were prepared and analyzed for luciferase or CAT and β-galactosidase activities as described (32).

DNA Binding Assays

A P^{32}-labeled oligonucleotide containing the human CYP3A4 PXRE (ER6, 5′-ATATGAACTCAAAGGAGGTCAGTG) motif was incubated for 20 min at room temperature with 1.5 μl of *in vitro* synthesized Δ^{ATG}hPXR, mRXRα (coupled transcription-translation (TNT) reticulocyte lysate system, Promega, Madison, WI), with or without purified GST or GST-mSHP, or 250-fold molar excess of competitor unlabeled ER6 oligonucleotide as previously reported (41). GST and GST-mSHP was purified from bacteria-expressed fusion protein and dialysis against 1× EMSA buffer [10 mM Tris (pH 8.0), 100 mM KCl]. mSHP and GST were stored at 1 mg/ml in 1× EMSA buffer complemented with 10% glycerol. DNA-protein complexes were resolved on a 4% polyacrylamide gel (30:1 acrylamide:bis-acrylamide) in 0.5× TBE (1× TBE = 89 mM Tris, 89 mM boric acid, and 2 mM EDTA). Gels were dried and subjected to autoradiography.

In Vitro Interaction

^{35}S-Methionine-labeled proteins were prepared by *in vitro* translation using the TNT-coupled transcriptional translation system according to the manufacturer's instructions (Promega). GST fusion proteins were expressed in the *Escherichia coli* BL21 strain and purified using glutathione-sepharose-4B bead affinity chromatography as suggested by the vendor (Pharmacia, Uppsala, Sweden). The beads were subsequently washed and resuspended in 20 mM Tris (pH 8.0), 100 mM NaCl, 0.1% Nonidet P-40 buffer (NETN). GST proteins bound to glutathione-sepharose were incubated with 5 μl of ^{35}S-methionine-labeled proteins in the presence of NETN buffer and 50 μM of indicated compound or 1% DMSO. After overnight incubation at 4 C with gentle agitation, agarose beads were extensively washed with NETN buffer and bound proteins were eluted in sample buffer and analyzed by SDS-PAGE. Gels were then stained with coomassie blue, incubated in an autoradiography enhancer (Dupont NEN, Boston, MA), dried and subjected to autoradiography at −70 C.

Primary Culture of Human Hepatocytes

Hepatocytes were prepared from lobectomy segments resected from adult patients for medically required purposes unrelated to our research program. The use of these human hepatic specimens for scientific purposes has been approved by the French National Ethics Committee. Hepatocytes were prepared and cultured according to the previously published procedure (42). The cells were plated into 60-mm plastic dishes precoated with collagen at 4×10^6 cells per plate in a total volume of 3 ml of a hormonally and chemically defined medium elaborated from a mixture of Williams' E and Ham F12 (1:1 in volume). Forty-eight hours after plating, cells were cultured in the presence or absence of CDCA (10–50 μM) for 72 h. Cells were then treated with DMSO or 5 μM RIF for 16 h.

Animals and Treatments

Male mice (C57/BL6 from Charles River Laboratories, L'Arbresle, France, BL3EV05919) were housed in a pathogen-free animal facility under a standard 12-h light, 12-h dark cycle. After 1 wk of acclimatization, mice were fed *ad libitum* for 3 d with standard rodent chow (diet-1820 (4.5% lipids) Harlan, Ganat, France) or supplemented in house with 1% CA. PCN was injected for two successive daily administrations of 40 mg/kg body wet, in corn oil. The mice were killed 24 h following the last PCN administration.

Total RNA Purification and Northern Blot

Total RNA was extracted from frozen mice liver tissues or human hepatocytes using Trizol reagent (GIBCO BRL, Cergy-Pontoise, France) according to the manufacturer's instructions and its purity was confirmed by spectrophotometry. For Northern blot experiments, 30 μg of total RNA were analyzed using $\alpha(^{32}P)$-dCTP-labeled CYP3A4 and rat GAPDH cDNA probes as previously described (32). Probe for SHP was obtained after *Sma*I/*Xho*I digestion and purification of the CDM8-mSHP plasmid. Probe for CYP7α (716 nucleotides) was generated by RT-PCR using the following primers 5′-TCCAGCGACTTTCTGGAGTT and 5′-AAAGGGACTGTGTG-GTGAGG (NM_000780). After PCR, the cDNA was cloned into pCR2-TOPO (Invitrogen, Cergy-Pontoise, France) and verified by sequencing. The signals were analyzed by quantifying the radioactivity with a PhosphoImager apparatus and ImageQuant software (both from Molecular Dynamics, Sunnyvale, CA).

Quantitative PCR

Quantification of GAPDH, CYP2B6, CYP2C9, PXR, and SHP mRNA was performed using the Roche Light Cycler apparatus. cDNA were synthesized from 1 μg of total RNA using the Superscript II first-strand synthesis system for PCR (Invitrogen) at 42 C for 60 min, in the presence of random hexamers. One tenth was used for PCR amplification. The following program was used: denaturation step 95 C, 8 min; 45 cycles of PCR (denaturation 95 C, 15 sec; annealing 65 C, 7 sec; elongation 72 C, 19 sec). In all cases, the quality of the PCR-product was assessed by monitoring a fusion step.

Sense and reverse primers were as follows, respectively: GAPDH: 5′-GGTCGGAGTCAACGGATTTGGTCG and 5′-CAAAGTTGTCATGGATGACC, CYP2B6: 5′-GGCCATACGGGAGGCCCTTG and 5′-AGGGCCCCTTGGATTTCCG, CYP2C9: 5′-TCCTATCATTGATTACTTCCCG and 5′-AACTGCAGTGTTTTCCAAGC, PXR: 5′-TCCGGAAAGATCTGTGCTCT and 5′-AGGGAGATCTGGTCCTCGAT, SHP: 5′-CCAATGATAGGGCGAAAGAA and 5′-GCTGTCTGGAGTCCTTCTGG, CAR: 5′-CCGTGTGGGGTTCCAGGTAG, and 5′-CAGCCAGCAGGCCTAGCAAC, CYP7α: 5′-CACCTTGAGGACGGTTCCTA and 5′-CGATCCAAAGGGCATGTAGT.

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