RIFAMPICIN INDUCTION OF CYP3A4 REQUIRES PREGNANE X RECEPTOR CROSS TALK WITH HEPATOCYTE NUCLEAR FACTOR 4α AND COACTIVATORS, AND SUPPRESSION OF SMALL HETERODIMMER PARTNER GENE EXPRESSION

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ABSTRACT:

Bile acids and drugs activate pregnane X receptor (PXR) to induce CYP3A4, which is the predominant cytochrome P450 enzyme expressed in the liver and intestine and plays a critical role in detoxifying bile acids and drugs, and protecting against cholestasis. The aim of this study is to investigate the molecular mechanism of PXR cross talk with other nuclear receptors and coactivators in regulating human CYP3A4 gene transcription. Rifampicin dose dependently induced the CYP3A4 but inhibited small heterodimer partner (SHP) mRNA expression levels in primary human hepatocytes. Rifampicin strongly stimulated PXR and hepatocyte nuclear factor 4α (HNF4α) interaction, and CYP3A4 reporter activity, which was further stimulated by peroxisome proliferators-activated receptor γ co-activator 1α (PGC-1α) and steroid receptor coactivator-1 (SRC-1) but inhibited by SHP. Mutation of the putative HNF4α binding site in the distal xenobiotic responsive element module did not affect CYP3A4 basal promoter activity and synergistic stimulation by PXR and HNF4α. Chromatin immunoprecipitation assays revealed that rifampicin-activated PXR recruited HNF4α and SRC-1 to the CYP3A4 chromatin. On the other hand, SHP reduced PXR recruitment of HNF4α and SRC-1 to the CYP3A4 chromatin. The human SHP promoter was stimulated by HNF4α and PGC-1α. Upon activation by rifampicin, PXR inhibited SHP promoter activity. Results suggest that PXR strongly induces CYP3A4 gene transcription by interacting with HNF4α, SRC-1, and PGC-1α. PXR concomitantly inhibits SHP gene transcription and maximizes the PXR induction of the CYP3A4 gene in human livers. Drugs targeted to PXR may be developed for treating cholestatic liver diseases induced by bile acids and drugs.

CYP3A4 is the most abundant cytochrome P450 enzyme expressed in the liver and small intestine and plays a crucial role in metabolism and detoxification of xenobiotics and endobiotics (Guengerich, 1999). CYP3A4 has very broad substrate specificity and metabolizes over 50% of clinical drugs. The expression levels of CYP3A4 vary widely among individuals (Lehmann et al., 1998). Compounds that activate human PXR (NR1I2) also induce CYP3A4 and drug metabolism activity (Bertilsson et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). The maximal induction of the CYP3A4 gene by PXR requires both the proximal ER6 and the distal XREM (Goodwin et al., 1999).

Lithocholic acid (LCA) is the most efficacious bile acid that activates PXR and induces CYP3A4 to catalyze 6-hydroxylation of LCA to hyodeoxycholic acid (Staudinger et al., 2001; Xie et al., 2001). Feeding LCA causes liver damage in Pxr null mice, but transgenic mice expressing a human PXR are protected from bile acid toxicity and cholestasis (Staudinger et al., 2001). PXR inhibits cholesterol 7α-hydroxylase (CYP7A1), the regulatory gene in bile acid synthesis, but induces organic anion transport protein 2, a sinusoidal Na⁺-dependent bile acid transporter (Staudinger et al., 2001). Nuclear receptors interact with their coactivators such as steroid receptor coactivator-1 (SRC-1) or corepressors and activate or inhibit their target genes. We reported previously that rifampicin-activated PXR interacts with hepatocyte nuclear factor 4α (HNF4α) and blocks HNF4α recruitment of peroxisome proliferator-activated receptor γ coactivator 1α (PGC-1α) and small heterodimer partner (SHP), small heterodimer partner; SRC-1, steroid receptor coactivator-1; XREM, xenobiotic responsive element module; dXREM, distal XREM; UAS, upstream activating sequence; TK, thymidine kinase; LBD, ligand-binding domain; RID, receptor-interacting domain; DBD, DNA-binding domain; a.a., amino acid; PCR, polymerase chain reaction; DMSO, dimethyl sulfoxide; EMSA, electrophoretic mobility shift assay; HA, hemagglutinin.
coactivator-1α (PGC-1α), and results in inhibition of the human CYP7A1 gene (Li and Chiang, 2004). HNF4α plays a vital role in liver development and regulation of bile acid synthesis, lipid homeostasis, and xenobiotic response (Stroup and Chiang, 2000; Hayhurst et al., 2001; Kamiya et al., 2003). Both PXR and HNF4α interact with PGC-1α, which is a nuclear receptor coactivator induced by glucagon and glucocorticoid to regulate gluconeogenesis and bile acid synthesis (De Fabiani et al., 2003; Puigserver et al., 2003; Shin et al., 2003). HNF4α induces PXR gene transcription in fetal liver and enhances PXR induction of CYP3A4 (Kamiya et al., 2003; Tirona et al., 2003).

It has been reported recently that SHP interacts with rifampicin-activated PXR and inhibits the CYP3A4 gene expression in vitro (Ourlin et al., 2003). SHP is a negative nuclear receptor that is induced by bile acid-activated farnesoid X receptor (FXR) and inhibits the CYP7A1 gene (Goodwin et al., 2000; Lu et al., 2000). Therefore, the bile acid-induced FXR/SHP pathway could counteract the bile acid activation of the CYP3A4 gene transcription by PXR. The physiological role of SHP in regulation of CYP3A4 needs to be further studied.

In this study, we investigated the roles of human PXR, HNF4α, and SHP on the regulation of human CYP3A4 gene transcription. Our results suggest that CYP3A4 is subjected to a complex regulation by nuclear receptors and coactivators, and that maximal induction of CYP3A4 gene expression requires concomitant inhibition of SHP gene transcription by PXR.

**Materials and Methods**

**Cell Culture.** Human hepatoblastoma HepG2 cells were obtained from The American Type Culture Collection (Manassas, VA). Primary human hepatocytes were isolated from human donors (HH1115, 22 years, male; HH1117, 68 years, female; HH1119, 29 years, female) and were obtained through the Liver Tissue Procurement and Distribution System (LTPADS) of the National Institutes of Health (S. Strom, University of Pittsburgh, Pittsburgh, PA). Cells were cultured and maintained as described previously (Li and Chiang, 2004).

**Plasmids.** Human CYP3A4/luciferase construct (p3A4-5′-dDR3/dER6/pER6-LUC) was obtained from Marie-Jose Vilarem (Institut Federatif de Recherche 24, 34293 Montpellier, France). This report contains an HNF4α binding site (DR1) and PXR binding sites (DR3 and ER6) in the distal XREM (ΔxREM, -7800 to -7600), which was linked to a 5′-flanking fragment containing a proximal ER6 (pER6) of the human CYP3A4 gene (Drocourt et al., 2002). A human CYP3A4/luc reporter (pH-298/Luc) was constructed as described previously (Li and Chiang, 2004). Human SHP/luciferase reporter construct (SHP-Luc) containing a 2-kilobase promoter sequence and expression plasmid (pCDM8-mShP) were provided by Yoontakg Lee (Baylor College of Medicine, Houston, TX). Expression plasmids for SHP (pCDNA3-HA-SHP), SRC-1 (pCR3.1-hSRC-1A), PGC-1α (pCDNA3/HA-PGC-1α), human RXRα (pDNA3-hRXRα), rat HNF4α (pCMX-HNF4α), and human PXR (pSG5-hPXR) were obtained from H. S. Choi (Chungnam National University, Gwangju, Korea), M. J. Tsai (Baylor College of Medicine), A. Krali (The Scripps Research Institute, La Jolla, CA), R. Evans (Scripps Research Institute), W. Chin (Eli Lilly Research Laboratories, Indianapolis, IN), and S. Kliewer (University of Texas Southwestern Medical Center, Dallas, TX), respectively. For mammalian hybrid assays, the reporter 5′XUS-TK-Luc contains five copies of the upstream activating sequence (UAS) fused upstream of a thymidine kinase (TK) minimal promoter and the luciferase reporter gene. The Gal4 and VP16 fusion plasmids contained the ligand-binding domain (LBD) of nuclear receptors or nuclear receptor-interacting domain (RID) of coactivators fused to Gal4-DBD (DNA-binding domain) or VP16-AD (activation domain) vector. Mammalian two-hybrid fusion plasmids used were: pCMX-VP16-HNF4α (a.a. 125–364) from D. Moore (Baylor College of Medicine); Gal4-hPXR-LBD (a.a. 107–434), VP16-1R (a.a. 959–780), and VP16-hPXR-LBD (a.a. 107–437) from A. Takeshita (Toranomon Hospital, Tokyo, Japan); and Gal4-PGC-1α (full-length) from A. Krali (Scripps Research Institute). Gal4-HNF4α fusion plasmids pBx-HNF4α-FL (full-length) was obtained from I. Talianis (Institute of Molecular Biology and Biotechnology Foundation for Research and Technology, Hellas, Herakleion Crete, Greece). For glutathione S-transferase (GST) fusion protein expression, pGEX-4T-1 plasmid encoding the GST protein was obtained from Amersham Pharmacia Biotech (Little Chalfont, Buckinghamshire, UK). The pET25a-GST-HNF4α plasmid encoding a fusion protein of GST with full-length human HNF4α was a kind gift from Todd Leff (Wayne State University, Detroit, MI). GST-hPXR-LBD was cloned by inserting PCR-amplified human PXR ligand-binding domain (a.a. 241–434) into pGEX-4T-1 BamHI/Xhol sites. GST-mShP containing full-length SHP was obtained from D. Moore (Baylor College of Medicine).

**Quantitative Real-Time PCR Assay.** Primary human hepatocytes were maintained in six-well plates. Cells were treated with increasing amounts of rifampicin (1–10 μM) (Sigma, St. Louis, MO) for 24 h. Total RNA was isolated from the cells using Tri-Reagent (Sigma) according to the manufacturer’s protocol. Reverse transcription reactions were performed using a RETROscript kit according to the manufacturer’s instructions (Ambion Inc., Austin, TX). For real-time PCR, samples were prepared according to the PCR Taqman Universal Master Mix 2X protocol (Applied Biosystems, Foster City, CA). Amplification of human ubiquitin C was used as an internal reference gene. Quantitative PCR analysis was conducted on the ABI 7500 System software. Relative mRNA expression was quantified using the comparative Ct (Ct) method according to the ABI manual. Assay-on-Demand PCR primers and Taqman MGB probe mix used were human CYP3A4 (Cat. No. Hs00604506_m1), human CYP7A1 (Cat. No. Hs00167982_m1), human SHP (Cat. No. Hs00222627_m1), and human ubiquitin C (Cat. No. Hs00824723_m1).

**Transient Transfection Assay.** HepG2 cells were grown to ~80% confluence in 24-well tissue culture plates. Reporter plasmid (0.2 μg) was transfected with expression plasmid (0.1 μg) using LipofectAMINE 2000 reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s protocol. The pcMV-galactosidase plasmid (0.05 μg) was transfected as an internal standard for normalizing the transfection efficiency in each assay. In some samples, empty expression vectors were added to equalize the total amounts of plasmid DNA transfected in each assay. Cells were treated with vehicle (DMSO) or 10 μM rifampicin in serum-free media for 40 h and assayed for luciferase reporter activity using the Luciferase Assay System (Promega, Madison, WI). Luciferase activity was normalized by dividing the relative light units by β-galactosidase activity. Each assay was done in triplicate and individual experiments were repeated at least three times.

**Site-Directed Mutagenesis.** Mutations (shown in Fig. 3B) were introduced into the HNF4α binding site in dXREM of CYP3A4/luciferase construct (p3A4-5′-dDR3/dER6/pER6-LUC) using the PCR-based QuickChange Site-directed Mutagenesis Kit (Stratagene, La Jolla, CA). Two complementary oligonucleotides containing the mutations were used as PCR primers. PCR were set up according to the manufacturer’s instructions using 50 ng of template DNA and 125 ng of primers. PCR cycling parameters were set as follows: denaturing at 95°C for 2 min, followed by 18 cycles at 95°C for 30 s, 52°C for 1 min, and 68°C for 18 min. The reaction mixture was digested by 0.1 μg of MspI for 2 h to remove the plasmid DNA and transformed into XL-Blue super competent cells (Strategene) for selection of mutant clones. Mutations were confirmed by DNA sequencing.

**Electrophoretic Mobility Shift Assay (EMSA).** Double-stranded synthetic probes containing the HNF4α-binding site were labeled with [32P]dATP by filling-in reaction using the Klenow fragment of DNA polymerase I. Nuclear receptor HNF4α was synthesized in vitro using Quick-coupled Transcription/Translation Systems (Promega) programmed with receptor expression plasmids according to the manufacturer’s instructions. EMSA was performed as described in detail previously (Li and Chiang, 2004).

**Immuno blotting Analysis.** HepG2 cells were cultured in 100-mm dishes to 80% confluence. Cells were transfected with pCDNA3-3A-SHPS plasmid (5 or 10 μg) and incubated in serum-free media for 40 h. Cell lysates were analyzed by SDS-polyacrylamide gel electrophoresis. Antibody against HA-tag (Santa Cruz Biotechnology, Santa Cruz, CA) were used for Western blotting and detected by an ECL Western blotting detection kit (Amersham Biosciences, UK).

**Chromatin Immunoprecipitation (ChIP) Assay.** HepG2 cells were grown in 100-mm culture dishes to 80% confluence. HA-PGC-1α (10 μg) or HA-SHPS plasmid was transfected into HepG2 cells. Cells were treated with 10 μM rifampicin or vehicle (DMSO) in serum-free media for 40 h. ChIP assays were performed using a ChIP Assay kit (Upstate Cell Signaling Solutions, Char
Rifampicin Induces CYP3A4 but Inhibits SHP mRNA Expression in Primary Human Hepatocytes. We assayed the effect of rifampicin on CYP3A4, SHP, and CYP7A1 mRNA expression levels in primary human hepatocytes. Relative mRNA expression (untreated control is set as 1) levels are plotted versus concentrations of rifampicin.

Results

Rifampicin Induces CYP3A4 but Inhibits SHP mRNA Expression in Primary Human Hepatocytes. We assayed the effect of rifampicin on CYP3A4, SHP, and CYP7A1 mRNA expression in primary human hepatocytes (Fig. 1). Rifampicin dose dependently induced CYP3A4 mRNA expression by almost 150-fold at 10 μM. Interestingly, rifampicin strongly reduced SHP mRNA levels at a concentration as low as 1 μM. This is in contrast to the induction of SHP expression by bile acids via activation of FXR. As a positive control, rifampicin strongly inhibited CYP7A1 mRNA expression as previously reported (Li and Chiang, 2004). These data suggest that rifampicin activates PXR to induce CYP3A4, but reduces SHP mRNA expression.

HNF4α, SRC-1, and PGC-1α Coactivate, whereas SHP Inhibits PXR Induction of Human CYP3A4 Gene Transcription. The marked induction of CYP3A4 by rifampicin prompted us to investigate the molecular mechanism of PXR induction of the human CYP3A4 gene. Nuclear receptor activity is enhanced by coactivators and inhibited by corepressors. SRC-1 and PGC-1α are known to enhance HNF4α and PXR trans-activation activity (Li and Chiang, 2004). We reported previously that rifampicin-activated PXR inhibited CYP7A1 gene transcription by interacting with HNF4α and blocking PGC-1α recruitment to CYP7A1 chromatin (Li and Chiang, 2004). We performed reporter assays to study the effect of HNF4α, PGC-1α, and SRC-1 on PXR regulation of the human CYP3A4 gene. We used a CYP3A4 reporter that contains a well characterized distal XREM (−7800/−7600, containing an HNF4α and two PXR binding sites) linked to a 5’-flanking fragment (−262/+11, containing a PXR site). Figure 2A shows that cotransfection of this CYP3A4-Luc reporter plasmid with PXR and RXRα expression plasmids in HepG2 cells stimulated the basal reporter activity by about 3-fold. Rifampicin (10 μM) further stimulated CYP3A4 reporter activity by 4-fold. Surprisingly, we found that HNF4α did not have any effect on the CYP3A4 reporter activity. Cotransfection with both HNF4α and PXR caused a 2.5-fold stimulation of the CYP3A4 reporter activity stimulated by PXR alone when rifampicin was added. Figure 2B shows that SRC-1 and/or PGC-1α stimulated PXR induction of CYP3A4 reporter activity by 2-fold in the absence of rifampicin. Addition of
Figure 3B shows that mutations of the HNF4α site in the CY3P4 reporter plasmid did not affect the basal reporter activity and the synergistic stimulation of reporter activity by PXR and HNF4α. These results suggested that HNF4α could stimulate CY3P4 reporter activity without binding to the CY3P4 gene. To further demonstrate that HNF4α could interact with PXR without binding to DNA, mammalian one-hybrid assay was performed. Figure 3C demonstrated that HNF4α could stimulate Gal4 reporter (5XUAS-Luc) activity induced by binding of Gal4DBD-PXR fusion protein to the UAS of the Gal4 DNA-binding domain. All these results support the hypothesis that HNF4α interaction with PXR synergistically stimulates CY3P4 gene transcription and that the putative HNF4α binding site in the CY3P4 gene may not be important in promoter function.

**SHP Inhibits CY3P4 Reporter Activity.** A previous study shows that SHP inhibits CY3P4 reporter activity and blocks PXR binding to DNA in EMSA (Ourlin et al., 2003). We studied the effect of SHP on CY3P4 reporter activity stimulated by PXR and HNF4α in HepG2 cells. Figure 4A shows that PXR inhibited CY3P4 reporter activity when activated in the presence of rifampicin. Figure 4B shows that SHP also inhibited CY3P4 reporter activity that was synergistically stimulated by PXR and HNF4α in the presence of rifampicin. However, our EMSA failed to confirm that SHP prevents PXR binding to the PXR-binding site in the CY3P4 gene (data not shown). This discrepancy may be due to the amount of SHP used in the assay. We used in vitro translated SHP, which typically contains nanogram quantities of translated proteins, whereas Ourlin et al. (2003) used 9 µg of GST-SHP fusion protein in EMSAs.

**Mammalian Two-Hybrid Assays of SHP Inhibition of PXR Interaction with Coactivators.** We then performed cell-based mammalian two-hybrid assays in HepG2 cells to study the effect of SHP on PXR interaction with HNF4α, SRC-1, and PGC-1α. Gal4-fusion proteins should bind to the Gal4 binding site in the reporter plasmid 5XUAS-TK-Luc. If a Gal4-fusion protein interacts with a VP16 activation domain-fusion protein, the reporter activity should be stimulated. Addition of a competitor protein may disrupt the interaction and reduce the reporter activity. This assay is very sensitive in detecting protein-protein interaction without interference by endogenous transcription factors. Figure 5A shows that Gal4-PXR strongly interacts with VP16-HNF4α, and rifampicin was required for the interaction. Addition of SHP strongly inhibited PXR/HNF4α interaction. Similarly, Gal4-SHP interacted with VP16-PXR in the presence of rifampicin, and HNF4α inhibited the interaction (Fig. 5B). Therefore, HNF4α and SHP competed for interaction with PXR. We did two-hybrid assays of Gal4-PXR interaction with VP16-SRC-1 or VP16-PGC-1α to test whether SHP inhibited PXR interaction with SRC-1 and PGC-1α. Figure 5C shows that Gal4-PXR interacted with VP16-SRC-1, but SHP only partially inhibited the interaction. In contrast, Gal4-PGC-1α interacted with VP16-PXR, but SHP did not affect their interaction (Fig. 5D). These results indicated that HNF4α and SHP competed for binding to PXR. However, SHP was unable to block PXR/PGC-1α interaction and only partially blocked PXR/SRC-1 interaction.

**GST Pull-Down Assays of PXR Interaction with HNF4α, SRC-1, PGC-1α, and SHP.** The GST pull-down assay is a cell-free assay for studying specific protein-protein interactions. GST-HNF4α (full-length) fusion protein or GST-hPXR (LBD) fusion protein strongly interacted with PXR or HNF4α, respectively, only when rifampicin was added to activate PXR (Fig. 6A). PXR interacted with SRC-1 in the absence of rifampicin, and addition of rifampicin enhanced their interaction. PXR interaction with PGC-1α does not require rifampicin. The degrees of ligand dependence for these coactivators to interact with PXR are consistent with mammalian two-hybrid assays shown in Fig. 5. We next performed GST pull-down assays to study the effect of rifampicin on rifampicin further stimulated reporter activity. The maximal induction (about 130-fold) of the CY3P4 reporter activity by rifampicin was achieved when PXR, HNF4α, SRC-1, and PGC-1α were transfected. It is noted that inclusion of HNF4α in the assay greatly enhanced rifampicin induction of the reporter activity, suggesting that PXR and HNF4αsynergy is important for induction of CY3P4. These data suggest that to maximize rifampicin induction of CY3P4 gene transcription requires HNF4α and coactivators.

**HNF4α Does Not Directly Regulate CY3P4 Gene Transcription.** Our result that HNF4α alone did not affect CY3P4 reporter activity is in contrast to a previous report that HNF4α stimulated CY3P4-Luc reporter activity (Tirona et al., 2003). EMSA, shown in Fig. 3A, confirmed that the putative HNF4α binding site in the DRREM of the CY3P4 gene was able to bind HNF4α as the oligonucleotide probes designed according to a canonical DR1 motif, and the HNF4α binding sites in the CYP8B1 and CYP7A1 genes. Excess of unlabeled DR1 probe was able to compete out HNF4α binding to the radiolabeled 8B1, 7A1, and 3A4 probes. We then performed site-directed mutagenesis to mutate the putative HNF4α binding site in the CY3P4 reporter plasmid. The mutant 3A4 probe did not bind HNF4α (data not shown).
SHP interaction with PXR and HNF4α. In vitro translated and 35S-labeled PXR or SHP was used to interact with GST-SHP or GST-HNF4α, respectively. As shown in Fig. 6B, rifampicin strongly enhanced PXR and SHP interaction. However, rifampicin did not affect SHP interaction with HNF4α as expected. We then added in vitro translated unlabeled-SHP or PXR to study their effect on interaction of 35S-labeled PXR or SHP with HNF4α, respectively. Results shown in the lower panels of Fig. 6B demonstrated that the amount of PXR or SHP that bound to GST-HNF4α was decreased. These results provided additional evidence that SHP, PXR, and HNF4α interacted and competed for binding to each other.

FIG. 3. Synergistic induction of CYP3A4 by HNF4α and PXR does not require HNF4α binding to the CYP3A4 gene promoter. A, EMSA of HNF4α binding to CYP3A4 dXREM. Probes containing a consensus HNF4α binding site (DR1) or HNF4α binding sites from CYP8B1 promoter (8B1), CYP7A1 promoter (7A1) and CYP3A4 dXREM (3A4) were labeled with [35S]dATP. Each Probe (5 × 10^4 cpm) and 5 µl of in vitro synthesized HNF4α protein were mixed and applied to each lane as indicated. A 100-fold excess of unlabeled DR1 probes was used as competitors. B, luciferase reporter constructs (0.2 µg) of wild-type (WT) and mutant (Mut) CYP3A4 containing mutations in the dXREM HNF4α binding site were transfected into HepG2 cells. Expression plasmids (0.1 µg) of PXR, RXRα, and/or HNF4α were coexpressed as indicated. Cells were treated with vehicle (DMSO) or 10 µM rifampicin for 40 h and harvested for luciferase activity assays as described under Materials and Methods. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment, and * indicates statistically significant difference from control, N = 3, p < 0.05. C, mammalian one-hybrid assay of HNF4α coactivation of PXR. HepG2 cells were transfected with 5XUAS-TK-Luc reporter (0.2 µg) and 0.1 µg of expression plasmids Gal4-PXR, PCDNA3, or HNF4α as indicated. Cells were treated with vehicle (DMSO) or 10 µM rifampicin for 40 h. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment, and * indicates statistically significant difference from control, N = 3, p < 0.05.
FIG. 5. Mammalian two-hybrid assays of the effect of SHP on the PXR interaction with HNF4α, SRC-1, and PGC-1α. HepG2 cells were transfected with 5XUAS-TK-Luc reporter (0.2 μg) and 0.1 μg of expression plasmids Gal4, Gal4-PXR, Gal4-SHP, Gal4-PGC-1α, VP16, VP16-HNF4α, VP16-PXR, VP16-SRC1, SHP, and HNF4α, as indicated. Cells were treated with vehicle (DMSO) or 10 μM rifampicin for 40 h. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment, and * indicates statistically significant difference from control, N = 3, p < 0.05.

A. PXR
B. GST

FIG. 6. GST pull-down assays of PXR interactions with HNF4α, SRC-1, PGC-1α, and SHP. A, effect of rifampicin on HNF4α, PXR, SRC-1, and PGC-1α interaction. DMSO or 10 μM rifampicin was added to the incubation mixture. B, Effect of SHP on HNF4α and PXR interaction. GST-SHP fusion or GST-HNF4α fusion was incubated with 5 μl of 35S-labeled, in vitro translated PXR or SHP, and/or unlabeled SHP or PXR as indicated. DMSO or 10 μM rifampicin was added to the incubation mixture as indicated. One microliter of the in vitro translated protein was used as input. GST pull-down assays were performed as described under Materials and Methods.
ChIP Assays of PXR and Coactivator Recruitment to CYP3A4 Chromatin. We then performed ChIP assays using HepG2 cells to study the effect of rifampicin on PXR recruitment of HNF4α, SRC-1, and PGC-1α to the CYP3A4 chromatin that contains PXR binding sites. The ChIP assay is considered as an in vivo assay of transcription factor interaction and coactivator recruitment in the native promoter context. Because the levels of PGC-1α are low in HepG2 cells, PGC-1α was overexpressed in HepG2 cells for ChIP assays. An antibody against PXR, HNF4α, SRC-1, or PGC-1α was used to immunoprecipitate the cross-linked DNA-protein complexes in HepG2 cells treated with rifampicin or vehicle (DMSO) as indicated for 40 h. ChIP assays were performed as described under Materials and Methods. DNA fragments containing distal XREM (left panel), proximal ER6 (middle panel), or 2nd intron (right panel) were PCR-amplified (illustrated at the top of figure) and analyzed on a 2% agarose gel. Normal IgG was used alone as nonimmune control; 10% of the total cell lysate was used as input.

ChIP Assays of PXR and Coactivator Recruitment to CYP3A4 Chromatin. We then performed ChIP assays using HepG2 cells to study the effect of rifampicin on PXR recruitment of HNF4α, SRC-1, and PGC-1α to the CYP3A4 chromatin. A (upper panels), HepG2 cells were transfected with 10 µg of PGC-1α expression plasmids. Anti-PXR, anti-HNF4α, anti-PGC-1α, or anti-SRC-1 antibodies were used to precipitate the DNA-protein complexes. B (lower panels), effect of SHP on the PXR recruitment of HNF4α, SRC-1, and PGC-1α to CYP3A4 chromatin. HepG2 cells were transfected with 10 µg of PGC-1α and SHP expression plasmids or pcDNA3 empty plasmid as control for 40 h. An antibody against PXR, HNF4α, SRC-1, or PGC-1α was used to immunoprecipitate DNA-protein complexes. Cells were treated with 10 µM rifampicin or vehicle (DMSO) as indicated for 40 h. ChIP assays were performed as described under Materials and Methods. DNA fragments containing distal XREM (left panel), proximal ER6 (middle panel), or 2nd intron (right panel) were PCR-amplified (illustrated at the top of figure) and analyzed on a 2% agarose gel. Normal IgG was used alone as nonimmune control; 10% of the total cell lysate was used as input.

PXR Inhibits SHP Gene Transcription. Since rifampicin strongly reduced SHP mRNA expression, we hypothesized that PXR might...
inhibit $SHP$ gene transcription. We and others reported previously that $HNF4\alpha/H9251$ binds to the human $SHP$ promoter and stimulates $SHP$ gene expression (Jung and Kullak-Ublick, 2003; Jahan and Chiang, 2005). Figure 8A shows that $HNF4\alpha/H9251$ and $PGC-1\alpha/H9251$ stimulated SHP reporter activity in HepG2 cells independent of rifampicin. However, $PXR$ strongly inhibited the SHP reporter activity stimulated by $HNF4\alpha/H9251$ and $PGC-1\alpha/H9251$ only when rifampicin was added. As a positive control, $PXR$ inhibited the CYP7A1 reporter activity stimulated by $HNF4\alpha/H9251$ and $PGC-1\alpha/H9251$ in the presence of rifampicin (Fig. 8B) (Li and Chiang, 2004). In complete contrast, rifampicin-activated $PXR$ markedly stimulated CYP3A4 reporter activity when $HNF4\alpha$ and $PGC-1\alpha$ also were transfected (Fig. 8C). These data support our previous finding that $PXR$ inhibits the $HNF4\alpha$ target gene $CYP3A4$ by blocking $HNF4\alpha$ recruitment of $PGC-1\alpha$ to $CYP7A1$ promoter (Li and Chiang, 2004). It appears that the same mechanism also regulates the human SHP promoter. In contrast, $PXR$ stimulates the $PXR$ target gene $CYP3A4$. Taken together, our data suggest that $HNF4\alpha$ enhances $PXR$ induction of $CYP3A4$ by protein-protein interaction.

**Discussion**

The ability to be strongly induced by a large variety of structurally unrelated compounds allows $CYP3A4$ to efficiently respond to elevated toxic metabolites such as bile acids and drugs and protects the liver against cholestasis induced by these compounds. Extensive studies in recent years have provided convincing evidence that $PXR$ is the key regulator of phase I drug-metabolizing enzymes, phase II bile acid and bilirubin conjugation enzymes, and phase III drug and metabolite transporters (Boyer, 2005). $PXR$ is the most important nuclear receptor that induces $CYP3A4$ to hydroxylate bile acids and drugs. However, most studies on $PXR$ regulation of $CYP3A4$ were performed in mouse models. In this study, we used the primary human hepatocyte and HepG2 models to study the molecular mechanism of human $PXR$ regulation of $CYP3A4$. Our real-time PCR assays revealed a remarkable 150-fold induction of $CYP3A4$ mRNA expression by rifampicin in human primary hepatocytes. In contrast, rifampicin treatment reduced $SHP$ and $CYP7A1$ mRNA expression. These data prompted us to study the mechanism of $PXR$ regulation of $CYP3A4$ and $SHP$ gene transcription. Our study revealed that interaction of $PXR$ with $HNF4\alpha/H9251$ and its coactivators, $PGC-1\alpha/H9251$ and $SRC-1$, contributes to the strong induction of $CYP3A4$ by rifampicin and that concomitant inhibition of $SHP$ gene expression by $PXR$ minimizes the inhibitory effect of $SHP$ and maximizes $PXR$ induction of $CYP3A4$. Several studies suggest the involvement of $HNF4\alpha$ in regulation of the $CYP3A4$, $CYP2C8$, and $CYP2C9$ genes by $PXR$ (Kamiya et al., 2003; Tirona et al., 2003; Chen et al., 2005; Ferguson et al., 2005). This study suggests that $HNF4\alpha$ may act as a coactivator of $PXR$ to recruit $PGC-1\alpha$ and $SRC-1$ to the $PXR$ target gene, $CYP3A4$. $PXR$ interacts strongly with $HNF4\alpha$, $SRC-1$, $PGC-1\alpha$, and $SHP$. $SHP$ may compete with $HNF4\alpha$ and $SRC-1$ for binding to $PXR$ and may repress $CYP3A4$ gene transcription (Fig. 9). Therefore, to maximize the induction of $CYP3A4$, $PXR$ must inhibit $SHP$ gene transcription.

This study also revealed a general mechanism for $PXR$ inhibition of $HNF4\alpha$-activated gene transcription. Previous studies suggest that $PXR$ inhibited $CYP7A1$ by blocking $HNF4\alpha$ recruitment of $PGC-1\alpha$ (Bhalla et
FIG. 9. A model of PXR, HNF4α, and coactivator regulation of human CYP3A4 gene in hepatocytes. PXR/RXRα binds to dXREM and pER6 motifs in the human CYP3A4 promoter. Rifampicin induces PXR interaction with HNF4α, which is coactivated by PGC-1α and SRC-1. The HNF4α binding site located upstream of dXREM is not important in regulation of CYP3A4. SHP is able to inhibit CYP3A4 gene transcription by blocking PXR and HNF4α interaction, and recruitment of PGC-1α and SRC-1 to CYP3A4 chromatin. SHP gene expression is induced by HNF4α, which binds to a DR1 motif in the SHP promoter. Rifampicin-activated PXR inhibits SHP transcription by blocking PGC-1α recruitment to SHP chromatin. As a consequence, SHP expression is reduced and CYP3A4 transcription is maximized.


References

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