PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine

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Abstract **Mitochondrial sterol 27-hydroxylase (CYP27A1) catalyzes oxidative cleavage of the sterol side chain in the bile acid biosynthetic pathway in the liver and 27-hydroxylation of cholesterol in most tissues. Recent studies suggest that 27-hydroxycholesterol (27-HOC) activates liver orphan receptor α (LXRα) and induces the cholesterol efflux transporters ABCA1 and ABCG1 in macrophages. The steroid- and bile acid-activated pregnane X receptor (PXR) plays critical roles in the detoxification of bile acids, cholesterol metabolites, and xenobiotics. The role of CYP27A1 in the intestine is not known. This study investigated PXR and CYP27A1 regulation of cholesterol metabolism in the human intestinal cell lines Caco2 and Ls174T. A human PXR ligand, rifampicin, induced CYP27A1 mRNA expression in intestine cells but not in liver cells. Rifampicin induced *CYP27A1* gene transcription, increased intracellular 27-HOC levels, and induced ABCA1 and ABCG1 mRNA expression only in intestine cells. A functional PXR binding site was identified in the human *CYP27A1* gene. Chromatin immunoprecipitation assays revealed that rifampicin induced the PXR recruitment of steroid receptor coactivator 1 to CYP27A1 chromatin. Cholesterol loading markedly increased intracellular 27-HOC levels in intestine cells. Rifampicin, 27-HOC, and a potent LXRα agonist, T0901317, induced ABCA1 and ABCG1 protein expression and stimulated cholesterol efflux from intestine cells to apolipoprotein A-I and HDL. This study suggests an intestine-specific PXR/CYP27A1/LXRα pathway that regulates intestine cholesterol efflux and HDL assembly.**—Li, T., W. Chen, and J. Y. L. Chiang. **PXR induces CYP27A1 and regulates cholesterol metabolism in the intestine.** ***J. Lipid Res.*** **2007.** 48: **373–384.**

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Mitochondrial sterol 27-hydroxylase (CYP27A1) is expressed in the liver, peripheral tissues, and macrophages. In the liver, CYP27A1 catalyzes the oxidative cleavage of the steroid side chain in the classic bile acid biosynthetic pathway and hydroxylation of cholesterol to 27-hydroxycholesterol (27-HOC) and 3β-hydroxy-5-cholestenoic acid in most tissues (1, 2). It has been suggested that CYP27A1 may play a role in defense against atherosclerosis by converting excess cholesterol to 27-HOC in macrophages. Oxidized cholesterols (oxysterols) may be transported to hepatocytes and converted to bile acids (3). This process is similar to the reverse cholesterol transport from peripheral tissues and macrophages to the liver for biliary excretion. Cholesterol-laden monocytes and macrophages infiltrate into the endothelium of the arterial wall and lead to the formation of foam cells, fatty streaks, and plaques and the development of atherosclerosis (4). The importance of CYP27A1 in the control of cholesterol homeostasis and protection against atherosclerosis is supported by the link between *CYP27A1* gene mutations and the accumulation of high levels of cholesterol and premature atherosclerosis in cerebrotendinous xanthomatosis patients (5).

Endogenously generated oxysterols, such as 20(*S*)-hydroxycholesterol, 22(*R*)-hydroxycholesterol, 24(*S*),25-epoxycholesterol, and 24-, 25-, and 27-hydroxycholesterol are low-affinity ligands of the liver orphan receptor α (LXRα) (6–8), which regulates a subset of genes involved in cholesterol synthesis, efflux, and transport and plays a crucial role in the control of lipid homeostasis (9–11). Cholesterol loading dose-dependently stimulates the production of 27-HOC and the induction of ABCA1 and ABCG1 expression in human macrophages, suggesting that CYP27A1 plays a role in activation of the LXRα pathway in response to cholesterol loading and that 27-HOC is an endogenous LXRα ligand in macrophages (12, 13). This conclusion is supported by two recent reports that ligands for the retinoic acid receptor, retinoid X receptor (RXR), and peroxisome proliferator-activated receptor γ

Abbreviations: apoA-I, apolipoprotein A-I; ChIP, chromatin immunoprecipitation; CYP27A1, sterol 27-hydroxylase; EMSA, electrophoretic mobility shift assay; 27-HOC, 27-hydroxycholesterol; LXRα, liver orphan receptor α; PPARγ, peroxisome proliferator-activated receptor γ; PXR, pregnane X receptor; PXRE, pregnane X receptor response element; RXR, retinoid X receptor.

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(PPARγ) synergistically stimulate CYP27A1 expression and the production of 27-HOC, which induces LXRα and its target genes, ABCA1 and ABCG1 (14, 15).

Pregnane X receptor (PXR; NR1I2) is activated by sterols, bile acids, and >60% of clinical drugs. Despite the high sequence identity, human PXR and rodent PXR have very different ligand selectivity. For example, rifampicin is a potent ligand for human PXR but not mouse PXR, whereas pregnenolone 16α-carbonitrile is a potent ligand for mouse PXR but not human PXR (16). PXR plays a crucial role in inducing the CYP3A, CYP2B, and CYP2C families of drug-metabolizing enzymes. CYP3A4 is the most abundant cytochrome P450 enzyme in human liver and intestine and metabolizes >50% of clinical drugs (16). Feeding lithocholic acid caused liver damage in *Pxr* null mice and wild-type mice, but transgenic mice overexpressing the human PXR were protected from lithocholic acid toxicity (17). Lithocholic acid could activate mouse PXR to repress CYP7A1 (18). These studies established the mouse PXR as a lithocholic acid sensor that regulates some genes involved in bile acid synthesis, transport, and metabolism (17, 19). This suggests that PXR plays a critical role in bile acid metabolism and protects against lithocholic acid toxicity in mouse liver; however, its roles in the human liver and intestine remain to be demonstrated.

Oxysterols and cholesterol metabolites are able to activate PXR and induce Cyp3a in rat and mouse hepatocytes (20, 21). A bile acid metabolite, 5β-cholestane-3α,7α,12α-triol, is a substrate of both Cyp3a11 and Cyp27a1 and has been identified as a PXR ligand in mouse liver (22, 23). This bile acid intermediate is efficiently metabolized by Cyp3a11 in mouse liver but is accumulated in human cerebrotendinous xanthomatosis patients. Furthermore, a recent study reports that *Pxr* null mice fed a diet containing high cholesterol and cholic acid levels developed acute hepatorenal failure, which suggests that PXR may play a role in the detoxification of cholesterol metabolites (24). These studies established a link between PXR and cholesterol metabolism and a possible role for the PXR regulation of CYP27A1 expression.

In this study, we investigated the potential role of human PXR in the regulation of the human *CYP27A1* gene in liver and intestine. Our results show that cholesterol and rifampicin induce *CYP27A1* gene expression and 27-HOC production in intestine but not in liver cells. Our results reveal an intestine-specific regulation of human CYP27A1 by PXR and suggest that PXR may play an important role in regulating HDL metabolism in the intestine.

MATERIALS AND METHODS

Cell culture

The human hepatoblastoma cell line HepG2 and the human colon adenocarcinoma cell lines Caco2 and Ls174T were obtained from the American Type Culture Collection (Manassas, VA). The cells were grown in a 1:1 mixture of Dulbecco's modified Eagle's medium and F-12 (50:50; Life Technologies, Inc., Gaithersburg, MD) supplemented with 100 U/ml penicillin G/streptomycin sulfate (Celox Corp., Hopkins, MN) and 10% (v/v) heat-inactivated fetal calf serum (Irvine Scientific, Santa Ana, CA). Primary human hepatocytes were isolated from human donors (HH1115, 22 year old male; HH1117, 68 year old female; HH1119, 29 year old female) and were obtained through the Liver Tissue Procurement and Distribution System of the National Institutes of Health (S. Strom, University of Pittsburgh, Pittsburgh, PA). Cells were maintained in Hepatocyte maintenance medium (Clonetics, Cambrex Bioscience, Walkersville, MD) supplemented with 10^{-7} M insulin and dexamethasone and used within 24 h after receiving.

Reporter and receptor expression plasmids

Human CYP27A1/Luc reporter constructs ph-1774CYP27A1/Luc, ph-992CYP27A1/Luc, ph-502CYP27A1/Luc, ph-223CYP27A1/Luc, and ph-147CYP27A1/Luc, containing different lengths of the human CYP27A1 promoter, were constructed previously (25). The CYP3A4 promoter/luciferase construct (p3A4-5′-dDR3/dER6/pER6) was provided by M. Vilarem (Institut Federatif de Recherche 24, France). Expression plasmids for RXR (pcDNA3-hRXR) and human PXR (pSG5-hPXR) were provided by R. Evans (Scripps Research Institute, La Jolla, CA) and S. Kliewer (University of Texas Southwestern Medical Center, Dallas, TX), respectively.

RNA isolation and quantitative real-time PCR

Primary human hepatocytes, HepG2, Caco2, and Ls174T cells were plated on six-well plates. After treatment with different reagents, total RNA was isolated from the cells using Tri-Reagent (Sigma, St Louis, MO) according to the manufacturer's protocol. Reverse transcription reactions were performed using the 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 ubiquitin C was used in the same reaction as an internal reference gene. Quantitative PCR analysis was conducted on the ABI 7500 System (Applied Biosystems). Relative mRNA expression was quantified using the comparative ΔCt method according to the ABI manual. Assay-on-Demand PCR primers and Taqman MGB probe mix (Applied Biosystems) used were as follows: human CYP27A1 (catalog number Hs00168003 m1); human CYP3A4 (catalog number Hs00604506 m1); ubiquitin C (catalog number Hs00824723 m1); human ABCA1 (catalog number Hs00194045 ml); and human ABCG1 (catalog number 00245154 ml).

Transient transfection assay

HepG2 and Caco2 cells were grown to ~80% confluence on 24-well tissue culture plates. In the transient transfection assay, human CYP27A1/luciferase reporter plasmid (1 μg) was cotransfected with PXR and RXRα expression plasmids (0.5 μg) using the calcium phosphate-DNA coprecipitation method. The pCMV β-galactosidase plasmid (0.25 μg) was transfected as an internal standard for normalizing the transfection efficiency in each assay. Four hours after transfection, cells were incubated in serum-free medium and treated with rifampicin in the concentrations indicated for 40 h. Cells were harvested for assay of luciferase reporter activity using the Luciferase Assay System (Promega). Luciferase activity was determined using a Lumat LB 9501 luminometer (Berthold Systems, Inc., Pittsburgh, PA) and 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. Data are plotted as means ± SD. Statistical analyses of treated versus untreated controls were performed using Student's *t*-test. $P < 0.05$ was considered statistically significant.

Site-directed mutagenesis

Mutations were introduced into the ph-1774CYP27A1/Luc construct using the QuikChange site-directed mutagenesis kit (Stratagene, La Jolla, CA). Two complementary oligonucleotides containing the mutations were used as PCR primers. PCRs 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, 55°C for 1 min, and 68°C for 18 min. The reaction mixture was digested by *Dpn*I for 2 h to remove the template DNA and transformed into XL1-Blue supercompetent cells (Stratagene) for selection of mutant clones. Mutations in each clone were confirmed by DNA sequencing.

Electrophoretic mobility shift assay

Nuclear receptors were synthesized in vitro using the Quick-Coupled Transcription/Translation Systems (Promega, Madison, WI) programmed with receptor expression plasmids according to the manufacturer's instructions and was described previously (18). Double-stranded synthetic probes for a PXR binding site (ER6) of the human *CYP3A4* gene and three putative pregnane X receptor response elements (PXREs; sequences shown in Fig. 6A below) of the human *CYP27A1* gene were labeled with [α^{32}P]dCTP and used for electrophoretic mobility shift assay (EMSA) and analyzed as described previously in detail (18).

Chromatin immunoprecipitation assays

Ls174T cells were grown in 100 mm culture dishes to 80% confluence. Cells were incubated in serum-free medium containing either vehicle (DMSO) or 20 μM rifampicin for 20 h. Chromatin immunoprecipitation (ChIP) assays were performed using the ChIP Assay kit (Upstate Biotechnology) according to the manufacturer's protocol and were described previously (18). Briefly, cells were cross-linked in 1% formaldehyde for 10 min and washed with ice-cold PBS containing protease inhibitors. Cells were scraped and incubated in 1% SDS lysis buffer for 30 min on ice and sonicated using a Branson Sonifier 250 with microtip setting 6 for 10 s pulses at 40% output for a total of 1.2 min to break the DNA into 0.2 to 2 kb fragments. Cell lysates were collected by centrifugation and diluted 10-fold in ChIP dilution buffer. Ten percent lysates were saved as "input." After preclearing the diluted cell lysate with protein A-agarose, DNA-protein complexes were precipitated by incubating the cell lysates with 10 μg of goat anti-PXR antibodies (catalog number 7737; Santa Cruz Biotechnology, Inc., Santa Cruz, CA) or anti-SRC1 (M-341; Santa Cruz Biotechnology) overnight followed by a 1 h incubation with protein A-agarose beads. Nonimmune IgG was used as a negative control. The beads were washed and eluted twice with 250 μl of ChIP elution buffer. The cross-links were reversed by adjusting NaCl concentration to 200 mM and incubated at 65°C for 4 h, followed by incubation in 0.04 μg/μl protease K for 1 h. A 268 bp fragment containing PXRE-A, a 109 bp fragment containing PXRE-B, a 101 bp fragment containing PXRE-C, a 215 bp fragment containing proximal ER6 of CYP3A4 (PXR binding site), and a 376 bp fragment from the CYP27A1 intron 1 region were amplified by PCR for 25 cycles and analyzed on a 2% agarose gel. The PCR primer pairs used to generate DNA fragments containing putative PXRE were as follows: PXRE-A site (−291/−23), CYP27-A-F, 5′-CGCCCAGAGTTCAGACCA-3′; CYP27-A-R, 5′-CTTTGGGTCGAGTGCTGAGT-3′; PXRE-B site (−980/−871), CYP27-B-F, 5′-GAAAGCCACACCTAGCAACTCA-3′; CYP27-B-R, 5′-CAGCTGGTTCTAACAGCATTTGA-3′; PXRE-C site (−1,509/−1,408), CYP27-C-F, 5′-TGGTCTAGGTTGGGTGCTGTG-3′; CYP27-C-R, 5′-GCCAGGCTGGTCTCAAACTC-3′; proximal ER6 region (−277/−62), CYP3A4-F, 5′-ATGCCAATGGCTCCACTTGAG-3′; CYP3A4-R, 5′-CTGGAGCTGCAGCCAGTAGCAG-3′; CYP27A1 intron 1 (+4,029/+4,405), CYP27intron1-F, 5′-GTGTGATGGCACAAACTTACAG-3′; CYP27intron1-R, 5′-GACATTGTGGTTGGCTGTTC-3′.

Assay of 27-HOC

The amount of 27-HOC produced by CYP27A1 was assayed by a HPLC-based method (26). Caco2 cells were cultured in T175 flasks until ~80% confluent and were then treated with cholesterol (50 μM) dissolved in (2-hydroxypropyl)-β-cyclodextrin solution [45% (w/v) in water; Sigma] and/or rifampicin (20 μM)

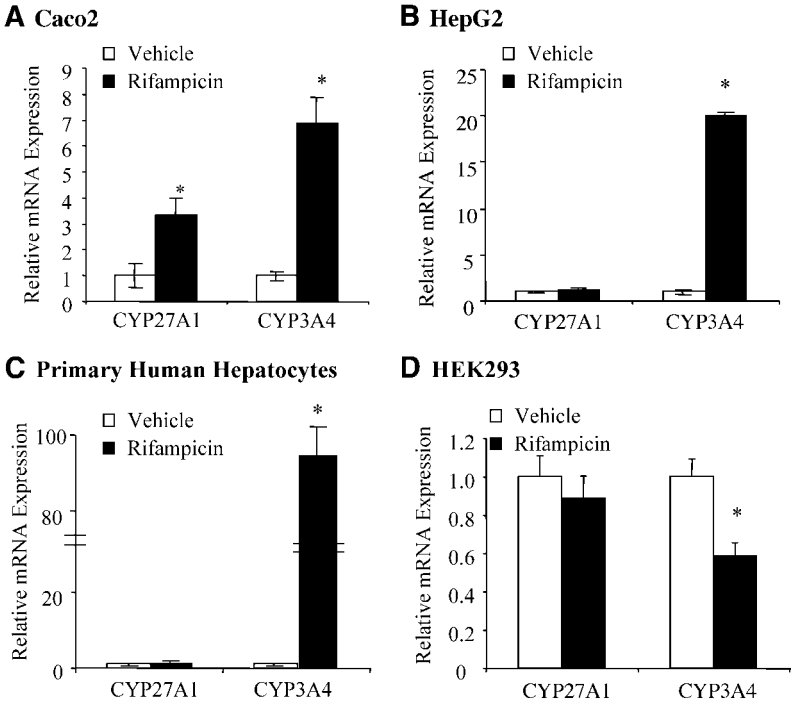

Fig. 1. Effect of rifampicin on sterol 27-hydroxylase (CYP27A1) mRNA expression. Caco2 cells (A), HepG2 cells (B), primary human hepatocytes (HH1119) (C), and HEK293 cells (D) were cultured on six-well plates. Cells were treated with either vehicle (DMSO) or 20 μM rifampicin and used to isolate mRNA for quantitative real-time PCR analysis of CYP27A1 and CYP3A4 mRNA expression. The mRNA expression levels are expressed as mRNA expression relative to untreated control levels as 1. Error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$).

for 72 h. Cells were then washed with ice-cold 1× PBS twice, resuspended in 1 ml of 1× reaction buffer (0.1 M phosphate, pH 7.5, 1 mM EDTA, and 2 mM DTT), and lysed by sonication. 20α-Hydroxycholesterol was added in each reaction as an internal recovery standard. Cholesterol oxidase (2 units; Sigma) was added to each reaction and incubated at 37°C for 30 min. The reaction mixtures were extracted twice with 4 ml of hexane, and the organic layers were evaporated to complete dryness. The samples were dissolved in 100 μl of sample buffer (5% isopropanol in dodecane) and analyzed on a normal-phase HPLC column (Pinnacle II silica 5 micron, 4.6 × 250 mm; Restek, Bellefonte, PA) using an isocratic mobile phase (96.5% hexane, 2.5% isopropanol, and 1% glacial acetic acid) at a flow rate of 1 ml/min. The metabolites were monitored at 240 nm. The amount of 27-HOC was determined from a 27-HOC standard curve, and intracellular 27-HOC concentrations were calculated by dividing the amount of 27-HOC by cell volume.

Cholesterol efflux assay

Cholesterol efflux was assayed according to an established method (27). Briefly, cells were cultured on 12-well plates until 50% confluent. Cells were then cultured in medium containing [^{3}H]cholesterol (3 μCi/ml; Sigma), cholesterol (50 μM), and 2 μg/ml ACAT inhibitor (SANDOZ 58-035; Sigma) for 24 h. Rifampicin (1 μM) or 27-HOC (10 μM) was then added, and cells were incubated for an additional 24 h. Medium was then removed, and cells were washed three times with PBS and incubated for 4–24 h in serum-free medium containing the same treatments. Medium was then removed, and cells were washed three times in PBS and incubated in serum-free medium containing 0.1% BSA, 50 μg/ml human HDL, or 15 μg/ml apolipoprotein A-I (apoA-I; Intracel, Frederick, MD). Aliquots of medium were taken out at each time point indicated. After 24 h, medium was removed and cells were washed three times with 1× PBS and lysed in 0.5 N NaOH. ^{3}H radioactivity in medium and cell lysate was measured by scintillation counting. Cholesterol efflux rate (%) was calculated by dividing radioactivity in the medium by total radioactivity in cell lysates plus medium. Assays were performed in triplicate, and results are expressed as means ± SD. Statistical analysis was performed by Student's *t*-test, with $P < 0.05$ indicating significant differences, control versus treated.

Immunoblot analysis

Ls174T or Caco2 cells were cultured in medium containing 50 μM cholesterol and treated with rifampicin (20 μM), 27-HOC

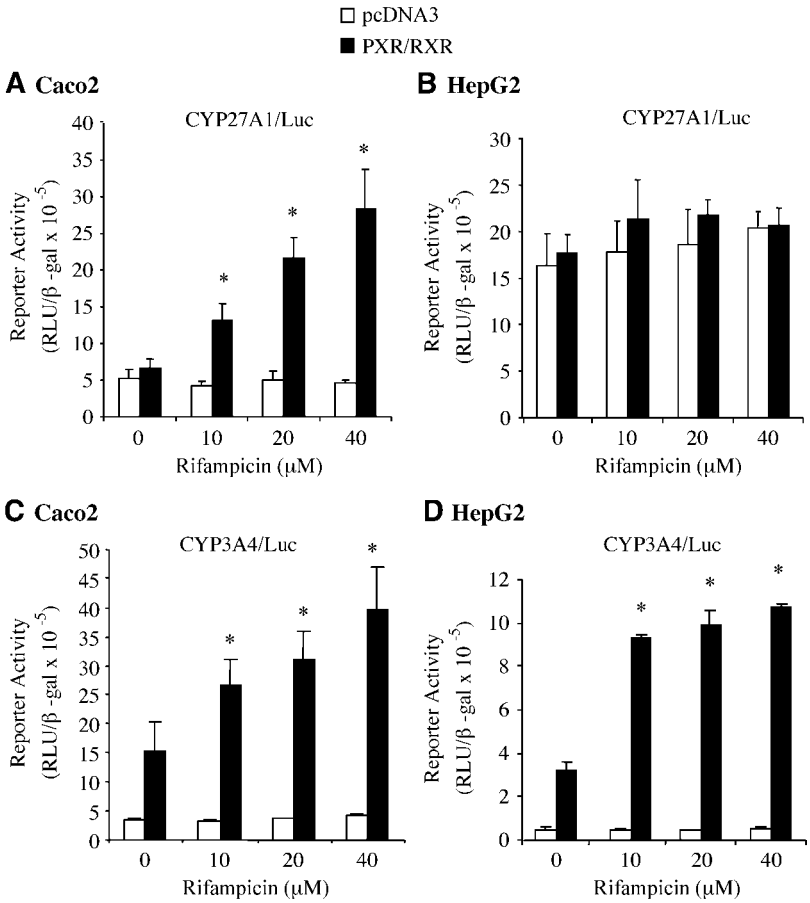

Fig. 2. Effect of rifampicin on human CYP27A1 promoter activities. Caco2 (A, C) and HepG2 (B, D) cells were cultured to ~80% confluence. A human CYP27A1/luciferase reporter, ph-1774CYP27/Luc (A, B), or a human CYP3A4/luciferase reporter, p3A4-5′dDR3/dER6/pER6 (C, D), was transiently transfected with pregnane X receptor (PXR) and retinoid X receptor α (RXRα) expression plasmids or pcDNA3 empty plasmid (control). Cells were treated with vehicle (DMSO) or rifampicin at the concentrations indicated and harvested for luciferase activity assays as described in Materials and Methods. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$). RLU, relative light units.

(10 μM), or T0901317 (1 μM) for 20 h as indicated. Total cell lysates were analyzed by SDS-PAGE. An antibody against ABCA1 (ab18180; Abcam, Cambridge, MA), ABCG1 (NB400-132; Novus Biologicals, Littleton, CO), or actin (sc-1615; Santa Cruz Biotechnology) was used for Western blotting and detected by the ECL Western blotting detection kit (GE Healthcare, Piscataway, NJ).

RESULTS

Rifampicin stimulated human CYP27A1 mRNA expression in intestine cells but not in human liver cells

Rifampicin is a specific and potent human PXR agonist that induces PXR target gene expression. We wanted to test whether PXR affects CYP27A1 expression in liver and intestine, two tissues in which PXR is highly expressed. Quantitative real-time PCR was performed to study the effects of rifampicin on CYP27A1 mRNA expression in human primary hepatocytes, human hepatocellular HepG2 cells, and human intestinal Caco2 cells. Rifampicin (20 μM) induced CYP27A1 mRNA expression by 3.3-fold in Caco2 cells (**Fig. 1A**) but did not affect CYP27A1 mRNA levels in primary human hepatocytes or HepG2 cells (Fig. 1B, C). Immunoblot analysis shows that PXR is expressed in intestine (Caco2 and Ls174T cells) and liver (HepG2 cells and human primary hepatocytes) but not in HEK293 cells (data not shown). CYP3A4 is expressed in liver and intestine and is a major PXR target gene. Therefore, we assayed the effect of rifampicin on CYP3A4 mRNA expression as a positive control. Figure 1A–C shows that rifampicin strongly induced CYP3A4 mRNA expression levels by 7-fold in Caco2 cells, by 20-fold in HepG2 cells, and by 90-fold in human primary hepatocytes. These results indicate that rifampicin induction of CYP27A1 mRNA expression is intestine-specific. To demonstrate that rifampicin induction of

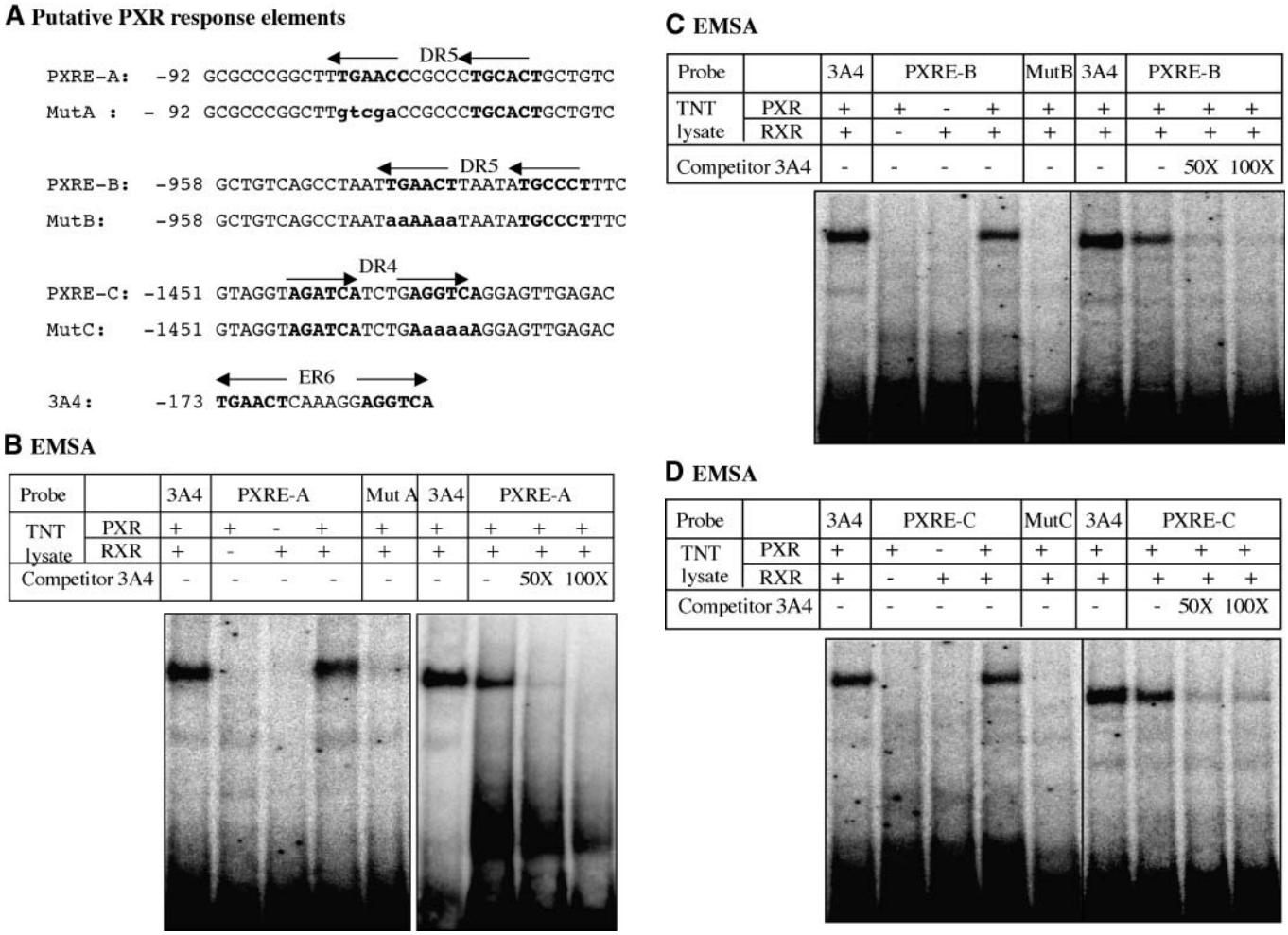

Fig. 3. Electrophoretic mobility shift assay (EMSA) of PXR binding to the human *CYP27A1* gene. A: Nucleotide sequences of the EMSA probes containing the putative pregnane X receptor response element (PXRE) in CYP27A1 (PXRE-A, PXRE-B, and PXRE-C) are illustrated. Arrows indicate the direction of hormone response element half-sites (boldface type). DR, direct repeat, ER, everted repeat. The nucleotide sequences of mutant probes (MutA, MutB, and MutC) of the PXR binding sites are indicated under the sequences of each wild-type probe (mutation in lowercase). Probe 3A4 was designed according to a well-characterized PXR/RXRα binding site (ER6) in the human *CYP3A4* gene. B: EMSA of the CYP27-A probe. C: EMSA of the CYP27-B probe. D: EMSA of the CYP27-C probe. α-^{32}P-labeled probes (5×10^4 cpm) and 5 μl of in vitro-synthesized proteins (TNT lysate) were mixed and applied to each lane as indicated. Excess unlabeled CYP3A4 probes or mutant probes were used as cold competitors.

CYP27A1 requires PXR, assays were performed in HEK293 cells that lack PXR. Figure 1D shows that rifampicin did not induce CYP27A1 mRNA expression in HEK293 cells. Rifampicin also did not induce CYP3A4 mRNA expression. The significant inhibition of CYP3A4 mRNA expression by rifampicin may be attributable to a PXR-independent mechanism (Fig. 1D).

PXR specifically stimulated human CYP27A1 reporter activity in Caco2 cells

We then performed reporter assays to study the possibility that PXR may regulate human *CYP27A1* gene transcription. Human CYP27A1 promoter/luciferase reporter (ph-1774CYP27A1/luc) activity was not stimulated by rifampicin without overexpressing PXR in Caco2 cells (**Fig. 2A**). In Caco2 cells overexpressing PXR and RXR, rifampicin dose-dependently stimulated CYP27A1 reporter activity (Fig. 2A). When reporter assays were performed in HepG2 cells, rifampicin did not stimulate CYP27A1 reporter activity, even when PXR and RXR were overexpressed (Fig. 2B). As a positive control, CYP3A4 reporter activity was strongly induced by rifampicin in both Caco2 and HepG2 cells overexpressing PXR and RXR (Fig. 2C, D). These results are consistent with the quantitative real-time PCR findings that PXR induces *CYP27A1* gene transcription in intestine cells but not in liver cells. We also tested the effects of the PPARα agonist WY14643 (10–100 μM), the PPARγ agonist troglitazone (1–10 μM), and T0901317 (0.01–1 μM) in cotransfection assays with their respective receptor plasmids. None of these receptors or their agonists significantly stimulated human CYP27A1 reporter activities in Caco2 cells (data not shown). These results suggest that PXR and its agonist specifically stimulate *CYP27A1* gene transcription in intestine cells.

Identification of putative PXR binding sites in the human CYP27A1 promoter

To identify PXREs in the human CYP27A1 promoter, we performed EMSA analyses of putative PXR binding sites (16). The PXR/RXR heterodimer has promiscuous binding activity to various direct repeat (DR3, DR4, and DR5) and everted repeat (ER6 and ER8) sequences. Three potential PXR binding sites containing either DR4 or DR5 were identified in the human CYP27A1 promoter and designated PXRE-A, PXRE-B, and PXRE-C (**Fig. 3A**). A canonical PXR/RXR binding site (ER6) from the human *CYP3A4* gene promoter was used as a positive control for EMSA. Mutant PXR binding sites (MutA, MutB, and MutC) were designed to test binding specificity (Fig. 3A). As shown in Fig. 3B–D, the PXR/RXR heterodimer was able to bind to all three probes. Excess unlabeled 3A4 probes or their respective mutant probes could abolish PXR/RXR bindings to these probes.

ChIP assay of PXR and coactivator recruitment to CYP27A1 chromatin

To further confirm that PXR binds to these putative PXR binding sites in CYP27A1 chromatin in vivo, ChIP assays were performed using Ls174T cell extracts. Cells were treated with an antibody against PXR or steroid receptor coactivator 1 to immunoprecipitate CYP27A1 chromatins. The fragments containing the PXR binding sites PXRE-A, PXRE-B, and PXRE-C on CYP27A1 were amplified by PCR and analyzed on agarose gels. A region containing a proximal ER6 in the human *CYP3A4* gene was amplified by PCR as a positive control. **Figure 4** shows that an antibody against PXR immunoprecipitated CYP27A1 chromatins containing these three putative PXREs. Rifampicin treatment (20 h) had no effect on PXR recruitment to CYP27A1 chromatins but strongly increased PXR recruitment of steroid receptor coactivator 1 to CYP27A1 chromatins. Similarly, rifampicin also increased PXR recruitment of steroid receptor coactivator 1 to CYP3A4 chromatin.

Reporter assay and mutagenesis analysis of a functional PXR site in the *CYP27A1* gene

To test whether these PXR/RXR binding sites are functional, we first used several CYP27A1 promoter/luciferase deletion constructs containing one, two, or three putative PXREs in transient transfection assays in Caco2 cells overexpressing PXR and RXR. As shown in **Fig. 5A**, rifampicin stimulated reporter activities of the CYP27A1 reporter constructs that contained PXRE-B (ph-1774CYP27A1/luc and ph-992CYP27A1/luc) but not the reporters without PXRE-B (ph-552CYP27A1/luc, ph-223CYP27A1/luc, and ph-147CYP27A1/luc). Deletion of the PXRE-C located upstream of PXRE-B did not affect the stimulatory effect of rifampicin. These data suggest that site B is necessary and sufficient to mediate rifampicin stimulation. To further confirm the function of these PXR binding sites in regulation of the *CYP27A1* gene, we introduced the same mu-

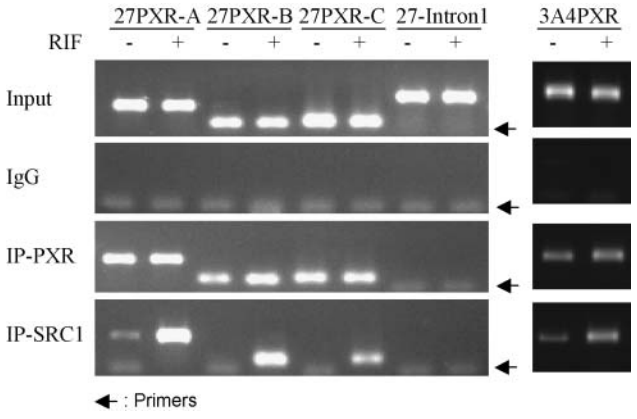

Fig. 4. Chromatin immunoprecipitation (ChIP) assays of PXR and steroid receptor coactivator 1 (SRC1) recruitment to CYP27A1 chromatin. Ls174T cells were treated with vehicle (DMSO) or 20 μM rifampicin (RIF) for 20 h. ChIP assays were performed as described in Materials and Methods. Anti-PXR or SRC1 antibodies were used to precipitate the DNA-protein complexes. The DNA fragment containing each PXR binding site on CYP27A1 (27PXR-A/B/C) or CYP3A4 (3A4PXR) was amplified by PCR and analyzed on a 1.5% agarose gel. Nonimmune IgG was used as a background control (IgG). The CYP27 intron 1 region (27-Intron1) from +4,029 to +4,405 was amplified by PCR as a control for immunoprecipitation (IP) specificity.

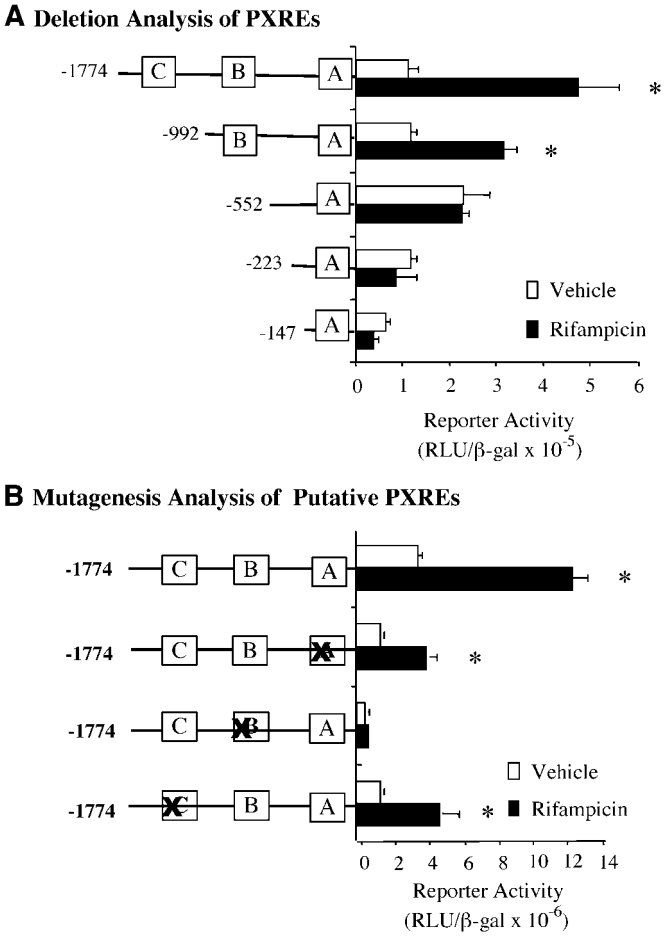

Fig. 5. Deletion and mutation analysis of PXR binding sites in the human CYP27A1 promoter. A: Human CYP27A1/luciferase reporters containing different length of the 5′ upstream sequence of the human *CYP27A1* gene were transiently transfected into Caco2 cells cotransfected with PXR and RXRα expression plasmids. Cells were treated with vehicle (DMSO) or 20 μM rifampicin for 40 h and harvested for luciferase activity assays as described in Materials and Methods. Boxes labeled A, B, and C represent the putative PXR binding sites shown in Fig. 3A. B: Wild-type ph-1774CYP27/luc reporter or mutant reporters with mutation in each PXR/RXRα binding site (MutA, MutB, and MutC, indicated by crosses) were transiently transfected into Caco2 cells cotransfected with PXR/RXR expression plasmids. Cells were treated with vehicle (DMSO) or 20 μM rifampicin and harvested for luciferase activity assays. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$). RLU, relative light units.

tations that abolished the PXR binding activity of three PXREs into the ph-1774CYP27/luc construct for reporter assays. Figure 5B shows that all three mutant reporters showed reduced basal activities compared with the wild-type reporter, suggesting that these elements may be important for basal promoter activity. Mutation of PXRE-B, but not PXRE-A and PXRE-C, abolished its responsiveness to rifampicin. Together, our data suggest that the PXR/RXR heterodimer binds to all three putative PXR binding sites, but only PXRE-B is responsive to the rifampicin induction of human CYP27A1 gene transcription. Other intestine factors (cofactors) may be involved in activation of the *CYP27A1* gene through the functional PXRE-B site.

Cholesterol and rifampicin increased intracellular 27-HOC levels in Caco2 cells

We then studied the physiological significance of the PXR induction of *CYP27A1* gene transcription in intestine cells. We first studied the effect of cholesterol loading and rifampicin on 27-HOC in Caco2 cells. **Figure 6A** shows that addition of cholesterol (50 μM, dissolved in β-cyclodextrin) to culture medium strongly increased 27-HOC levels by 18-fold in Caco2 cells. We calculated that the intracellular 27-HOC concentrations were ~1 μM in Caco2 cells cultured in medium without cholesterol

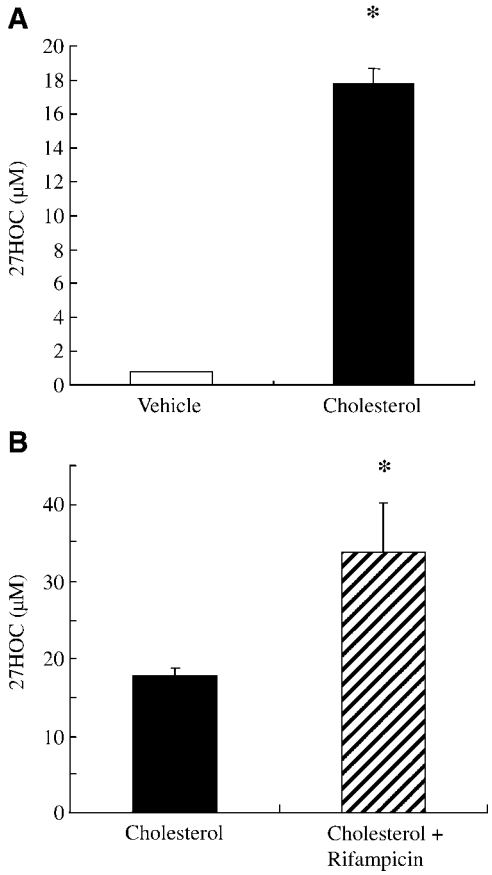

Fig. 6. Cholesterol loading and rifampicin increased 27-hydroxycholesterol (27-HOC) in Caco2 cells. A: Caco2 cells were cultured in T175 flasks until 80% confluent. Cells were treated with vehicle (β-cyclodextrin) or 50 μM cholesterol dissolved in β-cyclodextrin for 72 h. Cells were lysed, and 27-HOC concentrations were determined as described in Materials and Methods. The error bars represent the standard deviation from the mean of three individual experiments. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$). B: Caco2 cells were cultured in T175 flasks until 80% confluent. Cells were then cultured in medium containing 20 μM cholesterol and treated with vehicle (DMSO) or 20 μM rifampicin for 72 h. Cells were lysed, and 27-HOC concentrations were determined. Results were analyzed as described for A.

and ~18 μM in cholesterol-loaded Caco2 cells. Addition of rifampicin (20 μM) to cholesterol-loaded Caco2 cells further increased 27-HOC concentrations by ~2-fold (Fig. 6B). These results suggest that cholesterol loading activates CYP27A1 activity and drastically increases intracellular 27-HOC production in Caco2 cells and that rifampicin increases 27-HOC production by inducing CYP27A1 gene expression.

27-HOC induced the LXR target genes ABCA1 and ABCG1 in intestine cells

Because the intracellular 27-HOC concentration increased by cholesterol loading and rifampicin is sufficient to activate LXRα, we studied the effect of cholesterol and 27-HOC on the mRNA expression of a major LXRα target gene, ABCA1. It is believed that ABCG1 is expressed mainly in macrophages. The expression of ABCG1 in liver parenchymal and endothelial cells has been reported recently (28). Therefore, we also assayed the expression of ABCG1 mRNA in intestine cells. Quantitative real-time PCR analyses (**Fig. 7A**) show that cholesterol induced ABCA1 mRNA expression by 2- to 4-fold at 25–50 μM in Caco2 and Ls174T cells. Cholesterol induced ABCG1 mRNA levels by 6-fold at 50 μM. Figure 7B shows that 27-HOC induced ABCA1 and ABCG1 mRNA expression in a dose-dependent manner in Caco2 and Ls174T cells. The maximum induction of ABCG1 by 27-HOC was achieved at 10 μM, whereas ABCA1 was maximally induced at 50 μM.

Expression and induction of ABCA1 and ABCG1 in intestine cells

We then tested whether rifampicin treatment could further increase the mRNA expression of ABCA1 and ABCG1 in cholesterol-loaded Caco2 and Ls174T cells. As shown in **Fig. 8A**, rifampicin induced CYP27A1 mRNA expression by ~2.5-fold in cells loaded with 50 μM cholesterol. Rifampicin also significantly increased ABCA1 and ABCG1 mRNA expression levels in cholesterol-loaded cells (Fig. 8B, C). It should be noted that rifampicin did not induce ABCA1 and ABCG1 mRNA expression in Caco2 cells without loading of cholesterol (data not shown). This suggests that PXR does not directly induce ABCA1 and ABCG1 gene expression and that its effect is likely mediated through the induction of CYP27A1. A previous report shows that retinoids induce CYP27A1 expression in human macrophages loaded with cholesterol (14). We found that a retinoic acid receptor and RXR ligand, 9-*cis*-retinoic acid, significantly induced CYP27A1, ABCA1,

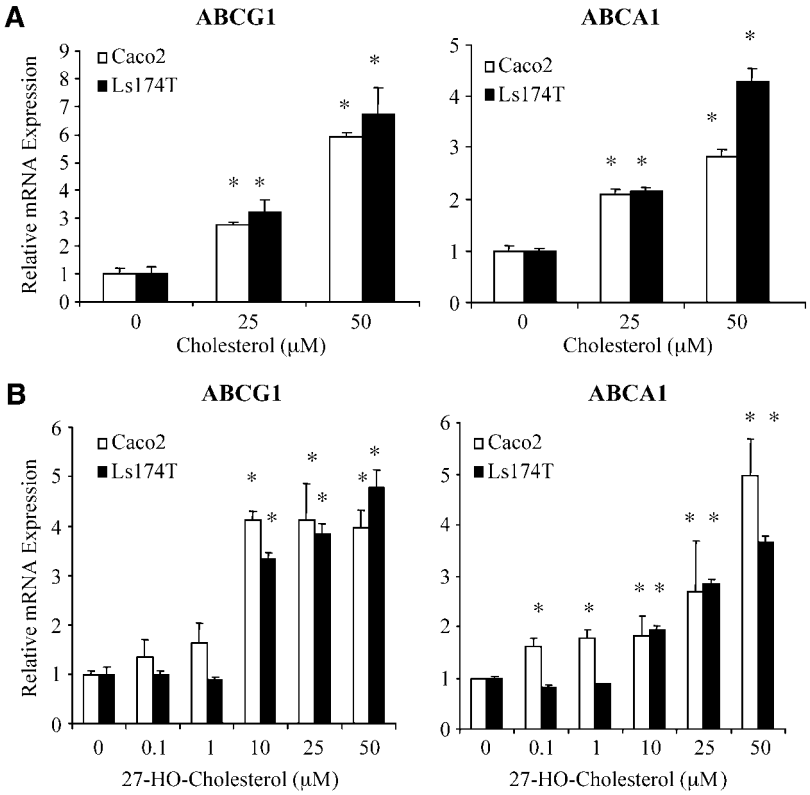

Fig. 7. Effect of 27-HOC and cholesterol loading on ABCA1 and ABCG1 mRNA expression in intestine cells. Caco2 and Ls174T cells were cultured to ~80% confluence on six-well plates. Cells were treated with an increasing amount of either cholesterol (A) or 27-HOC (B), and the ABCA1 and ABCG1 mRNA expression levels (relative to untreated control as 1) were determined by quantitative real-time PCR. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$).

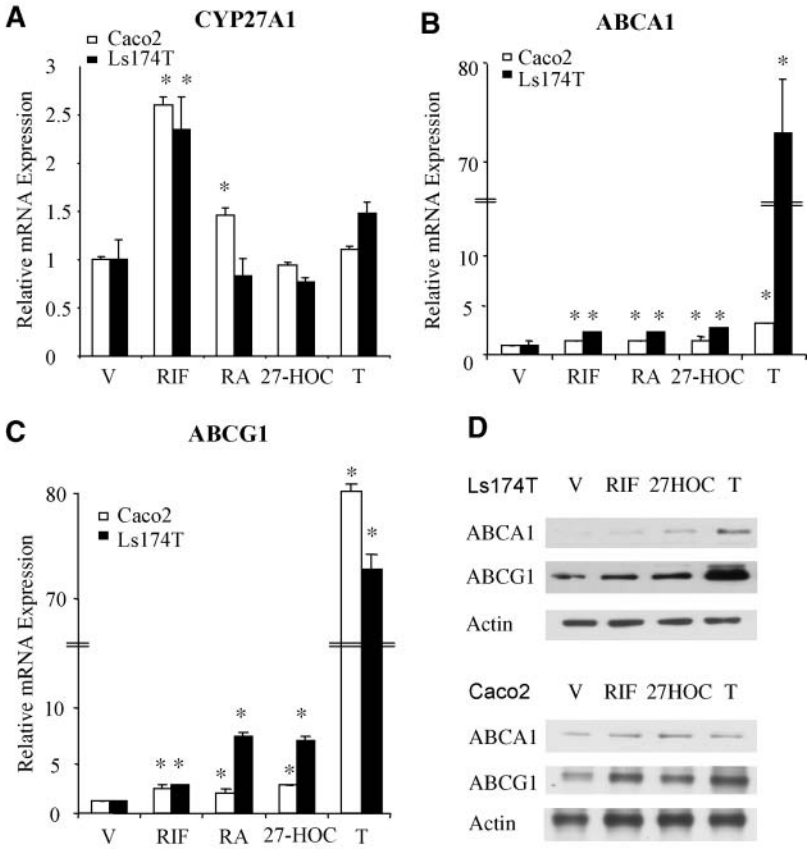

Fig. 8. Effects of PXR, retinoic acid receptor, RXR, and liver orphan receptor (LXR) agonists on CYP27A1, ABCA1, and ABCG1 mRNA expression. Caco2 and Ls174T cells were cultured to ~80% confluence on six-well plates. Cells were treated with vehicle (V; ethanol), 20 μM rifampicin (RIF), 1 μM 9-*cis*-retinoic acid (RA), 10 μM 27-HOC, or 1 μM T0901317 (T) for 40 h in medium containing 50 μM cholesterol. A–C: Relative mRNA expression levels of CYP27A1 (A), ABCA1 (B), and ABCG1 (C) were determined by quantitative real-time PCR. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.005$). D: Immunoblot analysis of ABCA1 and ABCG1 protein expression in Ls174T (upper panel) or Caco2 (lower panel) cells treated with rifampicin (20 μM), 27-HOC (10 μM), or T0901317 (1 μM).

and ABCG1 mRNA expression by 2.5-fold. However, 27-HOC (10 μM) and T0901317 (1 μM) did not affect CYP27A1 mRNA expression but strongly induced ABCA1 and ABCG1 mRNA levels by as much as 80-fold. Interestingly, T0901317 induction of ABCA1 in Ls174T cells was much stronger than that in Caco2 cells. A PPARγ-specific agonist, troglitazone, and a PPARα-specific agonist, WY14643, did not affect CYP27A1 expression (data not shown). These data suggest that CYP27A1 is a PXR target gene but not a LXRα target gene, whereas ABCA1 and ABCG1 are LXRα target genes but not direct PXR target genes.

To confirm that ABCG1 is expressed in intestine endothelial cells, we performed immunoblot analysis. Figure 8D shows that ABCG1 protein is expressed in both Ls174T and Caco2 cells. The expression of ABCA1 seems to be lower than that of ABCG1 in these two intestine cells. Both ABCG1 and ABCA1 protein expression levels were increased by rifampicin, 27-HOC, and T0901317. It should be noted that induction of ABCA1 and ABCG1 proteins by these compounds was much less than the induction of their mRNAs.

Effects of rifampicin and 27-HOC on intestinal cholesterol efflux to HDL

We then assayed cholesterol efflux from Ls174T cells to apoA-I or HDL. ^{3}H-labeled cholesterol was added to cells. Cells were washed, and apoA-I or HDL was added to assay the efflux of [^{3}H]cholesterol to culture medium. **Figure 9** shows that rifampicin significantly stimulated cholesterol efflux to apoA-I by 40% and to HDL by 25% at 6 h. 27-HOC also stimulated cholesterol efflux to a similar level, whereas T0901317 strongly stimulated cholesterol efflux to apoA-I or HDL by ~3-fold. The stimulation of cholesterol efflux rate by these compounds reflects their induction of ABCA1 and ABCG1 protein.

DISCUSSION

Despite some intense studies in recently years, the physiological function of CYP27A1 in extrahepatic tissues remains obscure. In this study, we reveal a novel role for CYP27A1 in HDL metabolism in the intestine. Cholesterol

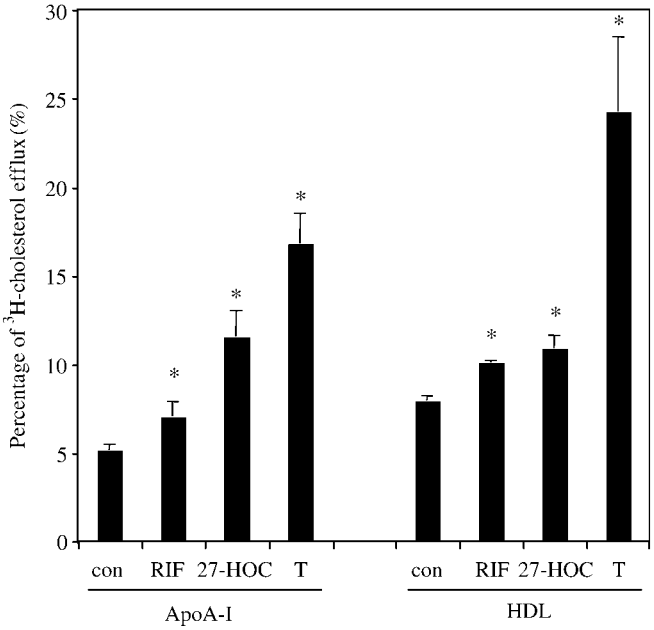

Fig. 9. Effect of rifampicin and 27-HOC on cholesterol efflux to apoA-I and HDL. ^{3}H-labeled cholesterol was added to Ls174T cells loaded with cholesterol. Rifampicin (RIF; 20 μM), 27-HOC (10 μM), or T0901317 (T; 1 μM) was added and incubated for 24 h. Medium was removed, and cells were washed. HDL or apoA-I particles were added as cholesterol acceptors to assay the [^{3}H]cholesterol efflux to the medium after 6 h. The error bars represent the standard deviation from the mean of triplicate assays of an individual experiment. * Significant difference, rifampicin treatment versus vehicle control (n = 3; $P < 0.05$).

loading activated CYP27A1 activity and markedly increased the intracellular concentration of 27-HOC to ~18 μM in intestine cells. This concentration of 27-HOC is similar to the 12–15 μM observed in macrophages loaded with cholesterol (14). Rifampicin-activated PXR induced *CYP27A1* gene transcription and further doubled intracellular 27-HOC levels. These concentrations of 27-HOC are sufficient to activate LXRα, which has an EC_{50} for 27-HOC of ~100–200 nM (12, 14). Our study suggests that 27-HOC is likely an endogenous LXRα ligand in intestine cells. The main function of CYP27A1 in intestine cells may be to induce LXR target genes, such as ABCA1 and ABCG1, which play critical roles in cholesterol efflux and HDL biogenesis. Interestingly, our results show that ABCG1 is highly regulated by 27-HOC and T0901317. As in macrophages, ABCG1 may also play a major role in cholesterol efflux and HDL assembly in the intestine.

Our data suggest that the human *CYP27A1* gene is not regulated by LXR and PXR in liver cells. This study clearly shows that CYP27A1 is a PXR target gene in intestine cells. Furthermore, rifampicin induces 27-HOC levels only in intestine cells preloaded with cholesterol, suggesting that CYP27A1 is mainly activated by the availability of its substrate cholesterol. This raises an interesting but intriguing question: why is the PXR regulation of CYP27A1 intestine-specific? Many genes are regulated in a tissue- and species-specific manner in response to stimuli. Nuclear receptors and coregulators integrate multiple signals to modify chromatin structure and regulate gene transcription (29). It is likely that different coregulators may be responsible for the tissue-specific regulation of the CYP27A1 gene by PXR.

CYP27A1 is located in the inner mitochondrial membrane which lack cholesterol. The alternative bile acid synthesis pathway initiated by CYP27A1 plays a minor role in bile acid synthesis in normal humans (30). To activate CYP27A1, cholesterol needs to be transported to mitochondria by steroidogenic acute regulatory proteins, which are expressed at very low levels in liver cells (31, 32). The intestine is exposed to very high levels of dietary cholesterol, which may induce steroidogenic acute regulatory proteins to transport cholesterol to mitochondria and activate CYP27A1.

Based on this study, we propose that the major role of CYP27A1 in intestine may be to produce oxysterols that activate LXRα to regulate intestinal cholesterol efflux and HDL assembly. A recent study has demonstrated that intestine ABCA1 contributes directly to ~30% of the steady-state plasma HDL pool (33). The role of ABCA1 and ABCG1 in HDL metabolism was recently defined (34, 35). ABCA1 effluxes cholesterol to apoA-I and lipid-poor HDL, whereas ABCG1 effluxes cholesterol to HDL particles in macrophages (36, 37). These two transporters synergistically stimulate cholesterol efflux in macrophages (38). Our quantitative PCR and immunoblot analyses demonstrate the expression of ABCG1 mRNA and protein in intestine cells (Fig. 8D). ABCG1 expression is highly induced by 9-*cis*-retinoic acid, 27-HOC, and T0901317 in intestine cells. These data suggest that ABCG1 may be expressed in the basolateral membrane of the enterocytes and play an important role in cholesterol efflux when enterocytes are exposed to high levels of dietary cholesterol.

This study reveals a new role for PXR in regulating the *CYP27A1* gene and HDL metabolism in intestine cells. Several bile acid and cholesterol metabolites produced in the bile acid synthesis and cholesterol/oxysterol synthesis pathways are potent endogenous PXR ligands (20–23). Bile acids are known to reduce HDL cholesterol, plasma apoA-I, and hepatic apoA-I mRNA expression in wild-type mice (39, 40). The inhibitory effect of bile acids is more pronounced in *Pxr* null mice and is attenuated in human PXR transgenic mice (41). It was suggested that PXR might antagonize the inhibitory effect of the bile acid receptor farnesoid X receptor on apoA-I mRNA expression and HDL metabolism and that bile acids may induce ABCA1 and ABCG1 gene transcription (42). Our finding that rifampicin-activated PXR regulates HDL metabolism is consistent with a recent report that the induction of CYP3A4 is correlated with increased plasma HDL and apoA-I levels and that rifampicin and other PXR agonists increase apoA-I expression and serum HDL-cholesterol levels in rodents (33). Our results may suggest an indirect mechanism for the PXR regulation of HDL metabolism via the induction of CYP27A1 and activation of the LXRα target genes ABCA1 and ABCG1 in intestine cells (41).

The intestine is exposed to high levels of cholesterol absorbed from the diet. Activation of PXR and CYP27A1

may be an adaptive response to a high-cholesterol diet to remove excess unesterified cholesterol in the intestine. The intestine is also exposed to high levels of bile acids, bacteria, and drugs. These agents are known to cause inflammation and acute-phase responses that increase cholesterol synthesis and cause cell injury. PXR may induce CYP27A1 to detoxify cholesterol metabolites and oxysterols.

In summary, our study reveals for the first time that PXR is a positive regulator of human CYP27A1 in the intestine and suggests that PXR may have a novel role in cholesterol detoxification in the intestine. Activation of PXR by endogenous cholesterol metabolites and drugs may feed-forward activate CYP3A4 and CYP27A1 to detoxify bile acids and cholesterol metabolites and may also promote cholesterol efflux and HDL synthesis. PXR agonists may have therapeutic potential for treating liver and cardiovascular diseases.

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