Threonine-290 Regulates Nuclear Translocation of the Human Pregnan X Receptor through Its Phosphorylation/Dephosphorylation by Ca\textsuperscript{2+}/Calmodulin-Dependent Protein Kinase II and Protein Phosphatase 1

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ABSTRACT

The human pregnane X receptor (hPXR) is recognized as a xenobiotic-sensing nuclear receptor that transcriptionally regulates the gene expression of drug-metabolizing enzymes and transporters. Our study elucidates the mechanism by which the localization of hPXR is regulated through threonine-290. A phosphomimetic mutation at threonine-290 (T290D) retained hPXR in the cytoplasm of HepG2, HuH6, and SW480 cells in vitro and the mouse liver in vivo even after treatment with rifampicin, and a phosphodeficient mutation (T290A) translocated from the cytoplasm to the nucleus as the wild-type hPXR. The amount of the unphosphorylated wild-type yellow fluorescent protein–hPXR fusion protein but not the T290A mutant increased on Phos-tag gels in response to stimulations with rifampicin and roscovitine, and a marked increase was observed in the unphosphorylated levels of the T290A mutant in nontreated cells. The Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II (CaMKII) inhibitor KN93 [2-[(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamino]] and transfection with anti-CaMKII small-interfering RNA (siRNA) enhanced the unphosphorylated levels of the wild-type protein. CaMKII directly phosphorylated the threonine-290 of hPXR, and the T290A mutant conferred resistance to CaMKII. The protein phosphatase (PP) inhibitor okadaic acid (100 nM) and transfection with anti-PP1 siRNA but not anti-PP2A siRNA led to reduced expression of CYP3A4 mRNA. After the rifampicin and roscovitine stimulations, PP1 was recruited to the wild-type hPXR but not the T290A mutant. These results suggest that phosphorylation at threonine-290 by CaMKII may impair the function of hPXR by repressing its translocation to the nucleus, and dephosphorylation by PP1 is necessary for the xenobiotic-dependent nuclear translocation of hPXR.

Introduction

The constitutive androstane receptor (CAR, nuclear receptor subfamily 1, group I, member 3 [NR1I3]) and pregnane X receptor (PXR, NR1I2) are predominantly expressed in the liver and gut, and have been identified as xenobiotic-sensing transcription factors that regulate the gene expression of phase I, II, and III metabolic enzymes and transporters that are involved in the metabolism and elimination of endogenous and exogenous substances, such as bilirubin, steroid hormones, and xenobiotics (Timsit and Negishi, 2007). CAR localizes to the cytoplasm of normal mouse hepatocytes without stimuli such as drug treatments, translocates to the nucleus in response to CAR activators including phenobarbital (Kawamoto et al., 1999), subsequently forms a heterodimer with the retinoid X receptor (RXR), and transactivates its target genes (Honkakoski et al., 1998). The threonine-38 of human CAR plays a central role in the initial step of CAR activation; the phosphorylation of threonine-38 by protein kinase C retains CAR in the cytoplasm, and its dephosphorylation by protein phosphatase (PP) 2A causes CAR to translocate to the nucleus (Yoshinari et al., 2003; Mutoh et al., 2009). An examination of the molecular mechanisms underlying the phenobarbital-induced activation of CAR revealed that phenobarbital bound to epidermal growth factor receptor and indirectly activated CAR by inhibiting epidermal growth factor receptor signaling; the phenobarbital treatment enables the unphosphorylated receptor for activated C kinase I to interact with PP2A and CAR, thereby promoting the dephosphorylation of CAR at threonine-38 (Mutoh et al., 2013; Meyer and Jirtile, 2013).

PXR activity can be modulated by translocation from the cytoplasm to the nucleus as well as the formation of heterodimers with RXR and

ABBREVIATIONS: ATP, Adenosine-5’-triphosphate; CaMKII, Ca\textsuperscript{2+}/calmodulin-dependent protein kinase II; CAR, constitutive androstane receptor; Ct\textsuperscript{c}, cycle threshold; CDK, cyclin-dependent kinase; DMSO, dimethylsulfoxide; DNAJC7, cytoplasmic CAR retention protein; GFP, green fluorescent protein; Go6983, 2-[(3-dimethylaminopropyl)-5-methoxyindol-3-y])maleimide; Hsp90, heat shock protein 90; hPXR, human PXR; KN93, 2-[(2-hydroxyethyl)-N-(4-methoxybenzenesulfonyl)]amino-N-(4-chlorocinnamyl)-N-methylbenzylamine; MBP, maltose-binding protein; PCR, polymerase chain reaction; PKA, protein kinase A; PKC, protein kinase C; PP, protein phosphatase; PXR, pregnane X receptor; Rp-8-Br-cAMPS, 8-bromoadenosine 3’,5’-cyclic monophosphorothioate; RXR, retinoid X receptor; siRNA, small-interfering RNA; UGT1A1, UDP-glucuronosyltransferase 1A1; YFP, yellow fluorescent protein.
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interactions with cofactors in the nucleus (Squires et al., 2004; Timsit and Negishi, 2007). Similar to CAR, PXR forms a complex with the cytoplasmic CAR retention protein (designated DNAJC7 in the National Center for Biotechnology Information database) and heat shock protein 90 (Hsp90) to maintain a cytoplasmic localization (Kobayashi et al., 2003; Squires et al., 2004); however, the molecular mechanism underlying nuclear translocation has not yet been fully elucidated. We previously demonstrated that cyclin-dependent kinase (CDK) 2 phosphorylated human pregnane X receptor (hPXR) at serine-350 to suppress binding with hXR and the coactivator and maintain the acetylation of the hPXR protein, thereby down-regulating hPXR activity (Sugatani et al., 2012). Because the transfection of anti-CDK2 small-interfering RNA (siRNA) was previously shown to elevate the levels of UDP-glucuronosyltransferase 1A1 (UGT1A1), CYP2B6, and CYP3A4 in HepG2 cells (Sugatani et al., 2010), we concluded that the expression of UGT1A1, CYP2B6, and CYP3A4 may be negatively regulated through a CDK2 signaling pathway linked to cell cycle progression in HepG2 cells. Lin et al. (2008) demonstrated that roscovitine activated hPXR-mediated CYP3A4 gene expression by inhibiting CDKs in a ligand-independent manner, and also that CDK2 negatively regulates the activity of hPXR in HepG2 cells. The phosphorylation of hPXR at specific amino acid residues and other post-translational modifications have been shown to modulate the cytoplasmic localization and nuclear activation affecting hPXR activity (Lichti-Kaiser et al., 2009a; Pondugula et al., 2009; Staudinger et al., 2011). Thus, while post-translational modifications to hPXR may be involved in the regulation of its function, less is known about the molecular mechanism regulating the translocation of hPXR to the cell nucleus.

PXR is a key transcriptional regulator of CYP3A4, and CYP3A4 is known to catalyze the oxidative metabolism of most administered drugs (Lehmann et al., 1998; DeLisle et al., 2011). UGT1A1, plays a critical role in the detoxification of potentially neurotoxic bilirubin by conjugating it with glucuronic acid for excretion in bile (Ostrow and Murphy, 1970) and also conjugates drugs and other xenobiotics (Radominska-Pandya et al., 1999; Tukey and Strassburg, 2000). We identified the gntR1 motif (-3382/-3367), which plays a central role in the expression of UGT1A1 and is mediated by both ICAR and hPXR (Sugatani et al., 2001, 2004, 2005a,b, 2008). Thus, in the present study, CYP3A4 and UGT1A1 were used as biomarkers of exogenously expressed hPXR activation. In a previous study (Sugatani et al., 2012), we demonstrated that a phosphomimetic mutation at the threonine-290 of hPXR suppressed the ligand-independent translocation of hPXR to the nucleus. We herein identified a phosphomimetic mutant of hPXR that abrogated ligand-induced nuclear translocation. Furthermore, we showed that Ca2+/calmodulin-dependent protein kinase II (CaMII) directly phosphorylated the threonine-290 of hPXR, which was retained in the cytoplasm, and PP1 was recruited to the hPXR-Hsp90 complex in rifampicin- and roscovitine-stimulated HepG2 cells. The regulated interaction of hPXR with CaMII and PP1 has provided novel insight into the molecular machinery by which hPXR is activated and translocated to the cell nucleus to induce the expression of target genes.

Materials and Methods

Roscovitine, okadaic acid, KN93 [2-[-N-(2-hydroxyethyl)]-N-(4-methoxybenzenesulfonyl)amino-N-(4-chlorocinnamyl)-N-methylbenzylamine], Go6983 [2-(1-(3-dimethylaminopropyl)-5-methoxyindol-3-yl)-3-(4-hydroxy-2,3-dihydroxy-5-yl)methane, and 8-bromoadenosine 3',5'-cyclic monophosphorothioate (Rp-8-Br-cAMPS) were purchased from Calbiochem (Darmstadt, Germany). Rifampicin was obtained from Sigma-Aldrich (St. Louis, MO). Adenosine-5'-triphosphate (ATP) was from Roche Diagnostics (Mannheim, Germany), and [γ-32P]ATP from PerkinElmer (Santa Clara, CA). All other chemicals and solvents were of analytical grade and obtained from commercial sources.

Plasmids. The hPXR expression vector was generously provided by Dr. Masahiko Negishi (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Mutations were induced in hPXR with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions (Sugatani et al., 2012). The primers used were: 5'-GTCACCTGAGATCCAAGCAACTGTTGTCACAACCGGGAAG-3', 5'-CTTGGTCAACTGAGATTCAACGCATGGTTCACCCGAGACTGAAG-3', and their complements for hPXR T290A and T290D, respectively. Vectors for the expression of hPXR, 2) Flag-tagged hPXR protein, 3) yellow fluorescent protein (YFP)-hPXR fusion protein, and 4) maltose-binding protein (MBP)-hPXR fusion protein were constructed by inserting the corresponding cDNAs, which were amplified by polymerase chain reaction (PCR) with the following primers: 1) 5'-tcaagcaagggtgagacccaaagaa-3' and 5'-gtttctctegaytctagcagctcatctgtgtgagatccgctccggg-3', 2) 5'-tcaagcaagggtgagacccaaagaa-3' and 5'-gtttctctegaytctagcagctcatctgtgagatccgctccggg-3', 3) 5'-tcaagcaagggtgagacccaaagaa-3' and 5'-gtttctctegaytctagcagctcatctgtgagatccgctccggg-3', respectively, into the BamHI and XhoI sites of pCR3-hRXR and the coactivator and maintain the acetylation of the hPXR protein, thereby down-regulating hPXR activity (Sugatani et al., 2012). Because the transfection of anti-CDK2 small-interfering RNA (siRNA) was previously shown to elevate the levels of UDP-glucuronosyltransferase 1A1 (UGT1A1), CYP2B6, and CYP3A4 in HepG2 cells (Sugatani et al., 2010), we concluded that the expression of UGT1A1, CYP2B6, and CYP3A4 may be negatively regulated through a CDK2 signaling pathway linked to cell cycle progression in HepG2 cells. Lin et al. (2008) demonstrated that roscovitine activated hPXR-mediated CYP3A4 gene expression by inhibiting CDKs in a ligand-independent manner, and also that CDK2 negatively regulates the activity of hPXR in HepG2 cells. The phosphorylation of hPXR at specific amino acid residues and other post-translational modifications have been shown to modulate the cytoplasmic localization and nuclear activation affecting hPXR activity (Lichti-Kaiser et al., 2009a; Pondugula et al., 2009; Staudinger et al., 2011). Thus, while post-translational modifications to hPXR may be involved in the regulation of its function, less is known about the molecular mechanism regulating the translocation of hPXR to the cell nucleus.

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for 10 seconds at 95°C, then two-step PCR for 40 cycles of 95°C for 5 seconds, followed by 60°C for 30 seconds. We used β-actin to normalize gene expression in all samples. Fold-induction values were calculated by subtracting the mean difference of gene and β-actin cycle threshold (Ct) numbers for each treatment group from the mean difference of gene and β-actin Ct numbers for the vehicle group and raising the difference to the power of 2 ($2^{\Delta\Delta Ct}$). All primers used for real-time PCR were listed in Table 1.

**Western Blot Analysis.** HepG2 cells (2 × 10^6 cells) seeded on 75-cm² flasks and cultured for 24 hours were transfected with expression vectors [pCR3 (10 μg), pcR3-hPXR (10 μg), the pCR3-hPXR mutant (10 μg), pCMV-DYKDQDDK-hPXR (10 μg), the pCMV-DYKDQDDK-hPXR mutant (10 μg), pEYPF-hPXR (10 μg), the pEYPF-hPXR mutant (10 μg), pMAL-hPXR (10 μg), or the pMAL-hPXR mutant (10 μg)] using TransIT-LT1 (Mirus Bio) according to the manufacturer’s instructions. Cells were given fresh medium 24 hours after transfection, further transfected with the expression vectors, and then cultured with rifampicin (5 × 10⁻⁶ M), roscovitine (5 × 10⁻⁶ M), or vehicle for an additional 24 hours unless stated otherwise. Treated and untreated cells were washed 3 times with ice-cold phosphate-buffered saline and lysed with a freshly prepared lysis buffer containing 50 mM Tris-HCl (pH 7.4), 1% NP-40, 0.25% sodium deoxycholate, 150 mM NaCl, 1 mM EDTA, 1 mM phenylmethylsulfonyl fluoride, 1 mM Na₃VO₄, 1 mM NaF, 1 mM benzamidine, 1 μg/ml aprotinin, and 1 μg/ml leupeptin at 4°C. The lysates were centrifuged at 110,000g for 10 minutes. Nuclear extracts and cytoplasmic fractions were prepared using a nuclear extract kit (Active Motif, Tokyo, Japan). Protein concentrations were determined using the DC protein assay (Bio-Rad Laboratories, Hercules, CA). Cytoplasmic fractions, nuclear extracts, or cell lysates (20 μg) were resolved by electrophoresis on a 5 to 20% polyacrylamide gel (ATTO Corporation, Tokyo, Japan), and electroblotted onto a polyvinylidene difluoride membrane (Millipore Corporation, Redford, MA). Membranes were blocked at 4°C overnight in blocking One (Nacalai Tesque, Kyoto, Japan) and probed for 1 hour with primary antibodies including the anti-DDDK (Flag) tag and anti-green fluorescent protein (GFP) from Medical & Biological Laboratories (Nagoya, Japan), anti-Hsp90, anti-phospho-CaMKII (T286), and anti-PP2A catalytic subunit protein from Cell Signaling Technology (Danvers, MA), anti-DNAJC7 from Abnova (Taipei, Taiwan), anti-Histone H1, anti-phospho-serine/threonine, anti-CaMKIIα, and anti-PPI-beta catalytic subunit (PP-Iβ) from Abcam (Cambridge, United Kingdom), and anti-α-tubulin from Oncogene Research Products (Boston, MA). Antigen-antibody complexes were detected using an appropriate secondary antibody conjugated to horseradish peroxidase [horseradish peroxidase-conjugated anti-rabbit, anti-goat, or anti-mouse immunoglobulin (Jackson ImmunoResearch Laboratories, West Grove, PA)] and visualized with an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, United Kingdom).

In Phos-tag SDS-PAGE, Phos-tag gels were prepared as 7% polyacrylamide SDS-PAGE gels with the addition of 5 × 10⁻⁵ M Phos-tag reagent and 1 × 10⁻⁴ M MnCl₂. After electrophoresis, Phos-tag gels was soaked in transfer buffer containing 1 × 10⁻³ M EDTA for 10 minutes, followed by another 10 minutes in buffer without EDTA to remove manganese ions from the gel before transferal to the polyvinylidene difluoride membrane.

**Small-Interfering RNA-Mediated Protein Knockdown.** We obtained siRNAs targeting the human PP1 catalytic subunit β isoform (NM_002709_stealth_456), human PP1 catalytic subunit β isoform control (NM_002709_stealth_control_456), human PP2A catalytic subunit alpha isoform (NM_002715_stealth_527), human PP2A catalytic subunit alpha isoform control (NM_002715_stealth_control_527), human CaMKII (Stealth siRNA and negative control (BLOCK-iT Stealth RNAi Negative Control) from Invitrogen/Life Technology (Carlsbad, CA). HepG2 cells cultured for 24 hours were transfected with siRNA duplexes (100 nM) using TransIT-siQUEST (Mirus Bio) according to the manufacturer’s instructions. Cells were given fresh medium 24 hours after transfection, and then retransfected with the siRNA duplexes for an additional 24 hours unless stated otherwise.

**In Vitro Kinase Assays.** MBP-hPXR wild-type and T290A mutant fusion proteins were expressed in NET Express Competent Escherichia coli (high efficiency) and purified using amylose resin according to the manufacturer’s protocols (New England Biolabs). Kinase assays for CaMKII (New England Biolabs), cAMP-dependent protein kinase (PKA), and protein kinase C (PKC) (Promega Corporation, Madison, WI) were performed according to the manufacturer’s specifications. Briefly, kinase reactions were performed in 30 μl of the kinase buffer [for CaMKII, 50 mM Tris-HCl (pH 7.5), 10 mM MgCl₂, 0.1 mM EDTA, 2 mM dithiothreitol, and 0.01% Brij 35; for PKA, 40 mM Tris-HCl (pH 7.4) and 5 mM MgCl₂; for PKC, 20 mM HEPES (pH 7.4), 3.4 mM CaCl₂, and 10 mM MgCl₂] containing 0.75 μg of MBP-fused protein, 100 μM ATP, and 0.12 MBq of [γ⁻³²P]ATP at 30°C for 5 and 10 minutes. Reactions were stopped by adding the same volume of 2x Laemmli sample buffer [125 mM Tris-HCl (pH 6.8), 4% SDS, 20% glycerol, 0.002% bromophenol blue, and 10% 2-mercaptoethanol] and samples were resolved by SDS-PAGE on a 5 to 20% gradient gel (ATTO Corporation) after heat denaturation. Phosphorylation of the substrate was visualized by autoradiography.

**Expression of Fluorescent Protein–Tagged Wild-Type hPXR, T290A, and T290D Mutant Proteins in Culture Cells In Vitro and Mouse Livers In Vivo.** To detect the localization of wild-type hPXR, T290A, and T290D mutant hPXR, HepG2, HuH6, and SW-480 cells (6 × 10⁴ /well) seeded on three-well glass coverslips (Teflon-printed glass slides; Eric Scientific Company, Portsmouth, NH) were transfected with expression vectors [pEYFP (0.2 μg), pEYPF-hPXR (0.2 μg), or the pEYPF-hPXR mutant (0.2 μg)] using TransIT-LT1 according to the manufacturer’s instructions. Cells were given fresh medium 24 hours after transfection, further transfected with the expression vectors, and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle for an additional

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**TABLE 1**

Sequences of the oligonucleotides used.

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24 hours. Plasmids were injected via the tail veins of male imprinting control region mice (age 4 weeks) using the TransIT-QR Hydrodynamic Delivery Starter Kit (Mirus Bio) according to the manufacturer’s instructions. Three hours later, the mice were injected intraperitoneally with 50 mg/kg of rifampicin or vehicle and then killed 5 hours later. Liver sections were prepared for confocal microscopy. Liver sections and cells were fixed with methanol, and the nuclein was stained with 3 μM 4,6-diamidino-2-phenylindole-2-HC (Dojindo Laboratories, Kumamoto, Japan). Liver sections and cells were then washed and mounted with Antifade reagent (Bio-Rad Laboratories). Fluorescence was visualized using a LSM510 confocal microscope (Carl Zeiss Microscopy GmbH, Jena, Germany). Regarding quantitation, the subcellular localization of YFP-hPXR and YFP-T290D mutant hPXR was scored in at least 100 cells.

**Coimmunoprecipitation.** HepG2 cells (2 × 10^6 cells) seeded on 75-cm² flasks and cultured for 24 hours were transfected with expression vectors [pEYFP (10 μg), pEYFP-hPXR (10 μg), or the pEYFP-hPXR mutant (10 μg)] using TransIT-LT1 (Mirus Bio) according to the manufacturer’s instructions. Cells were given fresh medium 24 hours after transfection, further transfected with the expression vectors, and cultured with rifampicin (5 × 10⁻⁶ M), roscovitine (5 × 10⁻⁶ M), or vehicle for an additional 24 hours. The cytoplasmic fraction (1.8–2.5 mg) unless otherwise stated was precleared with protein G agarose beads and rabbit IgG (Santa Cruz Biotechnology, Santa Cruz, CA) for 30 minutes at 4°C. 1) YFP-tagged proteins and Hsp90 were immunoprecipitated using an anti-DNAJC7 or anti-GFP antibody combined with the protein G agarose beads, 2) PP-1B, PP2A, and YFP-tagged proteins were immunoprecipitated using an anti-GFP antibody combined with the protein G agarose beads, and 3) Hsp90 and YFP-tagged proteins were immunoprecipitated using an anti-phospho-CaMKII (T286) antibody combined with the protein G agarose beads. The agarose beads were washed 3 times with Dulbecco’s phosphate buffer and subjected to SDS-PAGE on a 5 to 20% gradient gel (ATTO Corporation). The proteins were transferred to an immobilon-P transfer membrane (Millipore), and the blots were probed with antibodies as indicated.

**Statistics.** Values are expressed as the mean ± standard error. All data were analyzed using a one-way analysis of variance (ANOVA). The statistical significance of differences between groups was analyzed using ANOVA or an unpaired t test. P < 0.05 was considered statistically significant.

**Results**

Effects of Phosphomimetic and Phosphodeficient Mutations at Threonine-290 on the Subcellular localization of hPXR and UGT1A1 Expression in HepG2 Cells Stimulated with Rifampicin and Roscovitine. In a previous study (Sugatani et al., 2012), we found that the expression of UGT1A1 induced by the CDK2 inhibitor rosvitine was increased in HepG2 cells in the presence of exogenously expressed hPXR, whereas a phosphomimetic mutation at the threonine-57, threonine-290, serine-350, and threonine-408 of hPXR suppressed the activity induced by rosvitine. The phosphomimetic T290D mutant YFP-hPXR fusion protein but not the S350D or T408D mutant proteins was markedly retained in the cytoplasm and suppressed nuclear translocation after the treatment with rosvitine (Sugatani et al., 2012).

To determine whether phosphorylation at threonine-290 altered the nuclear translocation of hPXR and its transcriptional activity stimulated by the hPXR ligand rifampicin, we investigated the effects of phosphomimetic and phosphodeficient mutations at threonine-290 on nuclear translocation and UGT1A1 expression in HepG2 cells stimulated with rifampicin compared with roscovitine. Western blot analysis of the cytoplasmic fractions showed that the Flag-tagged hPXR T290D mutant protein levels were higher than those of the wild-type and T290A mutant proteins, that the T290D mutant proteins but not the wild-type or T290A mutant proteins were retained in the cytoplasm after the treatment with rifampicin and roscovitine, and also that translocation to the cell nucleus was suppressed (Fig. 1, A and B). No statistically significant change was observed in the expressed protein levels of hPXR after the mutation to either alanine or aspartic acid at threonine-290 (Fig. 1C), which suggested that the impaired function of the phosphomimetic mutant was not caused by its reduced expression. The T290D but not the T290A mutation attenuated the activity induced by rifampicin and roscovitine near to that in the pcR3 vector control group, in which the increase in the mRNA level of UGT1A1 induced by roscovitine appeared to result from endogenous nuclear receptor activation (Fig. 1D). Additionally, the T57D and S350D but not the T290D mutant proteins could translocate to the nucleus of rifampicin-treated HepG2 cells (Supplemental Fig. 1).

![Fig. 1. Western blot analysis of the Flag-tagged hPXR wild-type, T290A, and T290D mutant proteins and transactivation capacity of UGT1A1 by the mutated hPXR proteins in HepG2 cells. HepG2 cells cultured for 24 hours were transfected with expression vectors encoding the wild-type (WT) or mutant hPXR proteins.](image-url)
Because Hsp90 and DNAJC7 are known to be involved in maintaining the cytoplasmic localization of human and rodent PXR (Kobayashi et al., 2003; Squires et al., 2004; Sugatani et al., 2012), we investigated whether the hPXR T290D mutant formed a complex with Hsp90 and DNAJC7 in the cytoplasm. Immunoprecipitation with the anti-DNAJC7 antibody revealed that the T290D mutant in the cytoplasm formed a complex with DNAJC7 and Hsp90 (Fig. 2). The formation of the hPXR T290D mutant-DNAJC7-Hsp90 complex was not disrupted even after the treatments with rifampicin and roscovitine, which suggests that DNAJC7 and Hsp90 contributed to the retention of the T290D mutant protein in the cytoplasm.

Effects of a Phosphomimetic Mutation at Threonine-290 on Rifampicin-Responsive Nuclear Translocation of the hPXR Protein in Various Cells. To determine whether the hPXR T290D mutant exhibited specific subcellular localization to HepG2 cells, we examined rifampicin-sensitive nuclear translocation of the YFP-tagged hPXR T290D mutant protein in cells and tissues other than HepG2 cells (the human hepatoblastoma cell line HuH6, human colon carcinoma cell line SW480, and mouse liver). Although the YFP–wild-type hPXR protein translocated to the nucleus after the treatment with rifampicin, the YFP-hPXR T290D mutant protein was retained in the cytoplasm, and nuclear translocation of the mutant protein induced by rifampicin was suppressed in HuH6 and SW480 cells (Fig. 3). Furthermore, YFP-hPXR wild-type and T290D mutant proteins were expressed in the mouse liver in vivo. Most of the more than 100 cells examined showed nuclear translocation of the YFP-hPXR wild-type protein in rifampicin-exposed livers, whereas the YFP-hPXR T290D mutant protein was retained in the cytoplasm of mouse livers even after the treatment with rifampicin (Fig. 4). These results indicate that the expressed YFP-hPXR wild-type protein but not the T290D mutant protein is capable of translocating to the nucleus after rifampicin treatment in mouse livers.

Mobility Shift of Phosphorylated hPXR on Phos-Tag SDS-PAGE. To determine whether the threonine-290 of hPXR was phosphorylated in intact cells, using the mobility-shift technique on Phos-tag gels, we evaluated the responses of the YFP-hPXR or Flag-hPXR wild-type and T290A mutant to the treatment with rifampicin and roscovitine compared with standard gels as the loading control. As shown in Fig. 5A, the phosphorylated proteins of the YFP-hPXR wild-type and T290A mutant were detected on Phos-tag gels (Fig. 5, A and C). Furthermore, the amounts of the unphosphorylated YFP-hPXR and Flag-hPXR T290A mutant proteins in the vehicle-treated group detected on Phos-tag gels were markedly higher than those of the wild-type proteins, and the treatment with rifampicin and roscovitine did not further increase the amounts of the unphosphorylated T290A mutant proteins (Fig. 5). These results indicate that YFP-hPXR and Flag-hPXR T290A mutant proteins are less phosphorylated than the wild-type proteins.

Fig. 2. Binding of YFP-tagged hPXR wild-type, T290A, or T290D mutant proteins (A) and the Hsp90 protein (B) to the cytoplasmic DNAJC7 or the Hsp90 protein (C) to YFP-tagged hPXR wild-type, T290A, or T290D mutant proteins. HepG2 cells cultured for 24 hours were transfected with constructs expressing the wild-type (WT) and T290A and T290D mutant hPXR proteins. The medium was replaced 24 hours after transfection, and the cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M), roscovitine (5 × 10⁻⁶ M), or vehicle (DMSO) for an additional 24 hours. Cytoplasmic fractions (400 μg) were prepared and used for immunoprecipitation (IP) with the anti-DNAJC7 or anti-GFP antibody and Western blotting (WB) with the indicated antibodies. Ab, antibody; IgG HC, immunoglobulin G heavy chains.

Fig. 3. Nucleocytoplasmic distribution of the YFP-tagged wild-type and T290D mutant hPXR in HuH6 and SW480 cells. HuH6 and SW480 cells cultured for 24 hours were transfected with constructs expressing the wild-type and T290D mutant hPXR fusion proteins. The medium was replaced 24 hours after transfection, and the cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle (DMSO) for an additional 24 hours. (A) The subcellular localization of the YFP-hPXR protein was observed by fluorescence microscopy. DAPI, 4,6-diamidino-2-phenylindole-2-HC. (B) The percentage of cells with the YFP fusion protein expressed predominantly in the cytoplasm (Cyt), predominantly in the nucleus (Nuc), and predominantly in both the cytoplasm and nucleus (Cyt = Nuc) were determined using a fluorescent microscope. At least 100 cells expressing the fusion proteins were examined for each treatment.
The CaMKII Inhibitor KN93 and Anti-CaMKIIα siRNA Enhanced Dephosphorylated Levels of the hPXR Wild-Type Protein. Because threonine-290 is part of a consensus CaMKII phosphorylation sequence, we assessed whether KN93 (CaMKII inhibitor) and anti-CaMKIIα siRNA affected the phosphorylation of the hPXR protein in HepG2 cells. The dephosphorylated level of the hPXR wild-type protein was evaluated using the mobility-shift technique on Phos-tag gels, and then compared with standard gels as the loading control. As shown in Fig. 6, KN93 at 0.5 μM enhanced the dephosphorylated level of the hPXR wild-type protein in vehicle-treated cells as well as the dephosphorylated levels in rifampicin- and roscovitine-treated control cells.

To determine whether CaMKIIα contributed to the phosphorylation of hPXR, anti-CaMKIIα siRNA was introduced into HepG2 cells exogenously expressing hPXR and the dephosphorylated level of the hPXR protein was evaluated using the mobility-shift technique on Phos-tag gels (Fig. 7A) compared with standard gels as the loading control. Transfection with anti-CaMKIIα siRNA reduced the level of CaMKIIα mRNA to 0.30 ± 0.03 of the control level (n = 3) and CaMKIIα protein levels (Fig. 7B), and markedly elevated the dephosphorylated level of the hPXR wild-type protein in the vehicle-treated cells as the treatment with rifampicin elevated the dephosphorylated level of the hPXR wild type protein (Fig. 7). The dephosphorylated level of the hPXR T290A mutant protein in vehicle-treated cells as well as in rifampicin-treated cells was not affected by anti-CaMKIIα siRNA (Fig. 7). These results suggested that CaMKIIα may contribute to the phosphorylation of hPXR at threonine-290.

hPXR Was Phosphorylated at Threonine-290 by CaMKII In Vitro. hPXR contained two minimal recognition motifs R-X-X-S/T (amino acids 127–130 and 287–290) that could potentially be phosphorylated by CaMKII (White et al., 1998). A phosphomimetic mutation at serine-130 did not inhibit basal transcription activity (Lichti-Kaiser et al., 2009a), but that of threonine-290 markedly suppressed the activities of the hPXR (Fig. 1D). Additionally, because CaMKII, PKA, and PKC have similar recognition sites, we examined the effects of Rp-8-Br-cAMPS (PKA inhibitor) and Go6983 (PKC inhibitor) on the expression of UGT1A1 mRNA induced by rifampicin and roscovitine in HepG2 cells. The mRNA level of UGT1A1 induced by rifampicin or roscovitine was less in HepG2 cells simultaneously treated with Rp-8-Br-cAMPS compared with vehicle (saline), and it was slightly higher in the cells simultaneously treated with Go6983 compared with vehicle (dimethylsulfoxide [DMSO]) (Supplemental Fig. 2).

In vitro kinase assays with protein serine/threonine kinases such as CaMKII, PKA, and PKC, [γ-32P]ATP, and engineered MBP fusion proteins containing hPXR revealed that hPXR was phosphorylated by CaMKII, PKA, and PKC, as assessed by radiography (Fig. 8A). To identify the phosphorylated site(s), we investigated whether the hPXR T290A mutant protein was phosphorylated by these kinases, and found that mutating threonine-290 to alanine led to markedly lower phosphorylation levels by CaMKII than the wild-type hPXR protein;
PKA and PKC phosphorylated the hPXR T290A mutant protein to a similar extent as the hPXR wild-type protein (Fig. 8, A and B). Taken together, these results indicate that the threonine-290 of hPXR in the ligand-binding domain is the CaMKII-specific phosphorylation site.

Coprojection of hPXR and Hsp90 from the Cytoplasmic Fraction in HepG2 Cells Expressing the YFP-hPXR Wild-Type Protein with the Anti-Phospho-CaMKII Antibody. We used coimmunoprecipitation assays to investigate whether the hPXR wild-type protein interacted with activated CaMKII (which was phosphorylated at 286) in the cytoplasm. Immunoprecipitation with the anti-phospho-CaMKII (T286) antibody and normal rabbit IgG demonstrated that the YFP-hPXR wild-type protein in the cytoplasm coprecipitated with endogenous phosphorylated CaMKII, whereas the wild-type protein was not detected in the precipitate with normal rabbit IgG (Fig. 9). The formation of the hPXR wild-type–Hsp90–phosphorylated CaMKII complex suggests that activated CaMKII signaling contributes to the retention of hPXR in the cytoplasm.

Effects of Okadaic Acid, Anti-PP1 siRNA, and Anti-PP2A siRNA on the Induction of UGT1A1 and CYP3A4 mRNA Expression. The protein phosphatase 1/2A inhibitor okadaic acid at a high nM concentration (100 nM) suppressed the induction of UGT1A1 and CYP3A4 mRNAs by rifampicin and roscovitine in HepG2 cells but did not affect their induction at a low nanomolar concentration (5–10 nM) (Fig. 10). After the treatment with okadaic acid at 100 nM, the Flag-hPXR protein accumulated in the cytoplasm, whereas okadaic acid at high nanomolar concentrations appeared to reveal other functions than the protein phosphatase 1/2A inhibitor because of the elevation in the basal levels of UGT1A1 and CYP3A4 mRNAs (Fig. 10). Furthermore, to determine which protein serine/threonine phosphatases were required to induce CYP3A4 by rifampicin and roscovitine, anti-PP1 siRNA or anti-PP2A siRNA was introduced into HepG2 cells that did not exogenously express hPXR. As shown in Fig. 11, transfection with anti-PP1 siRNA and anti-PP2A siRNA reduced PP1 and PP2A mRNA levels, respectively. Transfection with anti-PP1 siRNA reduced the mRNA level of CYP3A4 induced by rifampicin, whereas transfection with anti-PP2A siRNA did not (Fig. 11). These results suggest that PP1 contributed to rifampicin-mediated gene expression.
Coprecipitation of PP1 but Not PP2A from the Cytoplasmic Fraction in HepG2 Cells Stimulated by Rifampicin and Roscovitine with the Anti-GFP Antibody. To investigate whether PP1 could interact with the hPXR-Hsp90 complex in the cytoplasmic fraction of HepG2 cells stimulated with rifampicin and roscovitine, PP1 and PP2A were coprecipitated from the cytoplasmic fraction of HepG2 cells expressing YFP-hPXR wild-type and T290A mutant proteins using the anti-GFP antibody, or expressing Flag-tagged hPXR wild-type and T290A mutant proteins using the anti-Flag antibody. The anti-GFP antibody coprecipitated the PP1-beta catalytic subunit and Hsp90 from the rifampicin- and roscovitine-stimulated HepG2 cytoplasmic fraction, and PP2A catalytic subunit alpha was not detected in the precipitates (Fig. 12). The Flag-tagged antibody also coprecipitated the PP1-beta catalytic subunit from the roscovitine-stimulated HepG2 cytoplasmic fraction (Supplemental Fig. 3). Furthermore, the PP1-beta catalytic subunit was not in the precipitates with normal rabbit IgG, which indicated that PP1 recruited the hPXR-Hsp90 complex in response to the rifampicin and roscovitine stimulations.

Coprecipitation of PP1 but Not PP2A from the Cytoplasmic Fraction in HepG2 Cells Stimulated by Rifampicin and Roscovitine with the Anti-GFP Antibody. To investigate whether PP1 could interact with the hPXR-Hsp90 complex in the cytoplasmic fraction of HepG2 cells stimulated with rifampicin and roscovitine, PP1 and PP2A were coprecipitated from the cytoplasmic fraction of HepG2 cells expressing YFP-hPXR wild-type and T290A mutant proteins using the anti-GFP antibody, or expressing Flag-tagged hPXR wild-type and T290A mutant proteins using the anti-Flag antibody. The anti-GFP antibody coprecipitated the PP1-beta catalytic subunit and Hsp90 from the rifampicin- and roscovitine-stimulated HepG2 cytoplasmic fraction, and PP2A catalytic subunit alpha was not detected in the precipitates (Fig. 12). The Flag-tagged antibody also coprecipitated the PP1-beta catalytic subunit from the roscovitine-stimulated HepG2 cytoplasmic fraction (Supplemental Fig. 3). Furthermore, the PP1-beta catalytic subunit was not in the precipitates with normal rabbit IgG, which indicated that PP1 recruited the hPXR-Hsp90 complex in response to the rifampicin and roscovitine stimulations.

Fig. 9. Coprecipitation of the YFP-hPXR fusion protein from the HepG2 cytoplasmic proteins with the anti–phospho-CaMKII (T286) antibody. HepG2 cells cultured for 24 hours were transfected with constructs expressing the YFP-hPXR wild-type fusion protein. The medium was replaced 24 hours after transfection, and the cells were retransfected and then cultured for an additional 24 hours. The cytoplasmic proteins prepared from these cells (1.8 mg each) were precipitated with an anti–phospho-CaMKII (T286) or normal rabbit IgG antibody. The resulting bound proteins were subjected to Western blot analysis with anti-GFP (A) and anti-Hsp90 (B) antibodies. Ab, antibody; IgG HC, immunoglobulin G heavy chains; IP, immunoprecipitation; WB, western blotting.

Fig. 10. Effects of okadaic acid on the expression of UGT1A1 (A) and CYP3A4 (B) mRNAs and the hPXR protein (C) in HepG2 cells expressing the hPXR protein. HepG2 cells cultured for 24 hours were transfected with expression plasmids for hPXR. The medium was replaced 24 hours after transfection, and the cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M, gray bar), roscovitine (5 × 10⁻⁶ M, closed bar), or vehicle (DMSO, open bar) in the presence of okadaic acid at the indicated concentrations for an additional 24 hours. (A and B) The mRNA levels in cells treated with vehicle were taken as one. Data presented are the average ± S.E. for three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 versus each group in the absence of okadaic acid; *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle-treated groups in the presence of okadaic acid at each concentrations. (C) The treatment with okadaic acid (1 × 10⁻⁷ M) led to the accumulation of the hPXR protein in the cytoplasmic fraction.

Fig. 11. Effects of anti-PP1 and anti-PP2A siRNA transfection on the expression of CYP3A4 mRNA. HepG2 cells cultured for 24 hours were transfected with anti-PP siRNA (1 × 10⁻⁷ M) or mock-transfected. The medium was replaced 24 hours after transfection, and the cells were retransfected with anti-PP siRNA (1 × 10⁻⁷ M) or mock-transfected and then cultured with rifampicin (5 × 10⁻⁶ M, open bar) or vehicle (DMSO, closed bar) for an additional 24 hours. The mRNA levels of PP1 (A), PP2A (B), and CYP3A4 (C) in cells cultured for 72 hours (control group) were taken as one. Data presented are the average ± S.E. for three experiments. ***P < 0.001 versus the mock-transfected and vehicle-treated groups; ###P < 0.001 versus the mock-transfected and rifampicin-treated groups.
PXR, a member of the nuclear receptor superfamily, was originally characterized as a xenobiotic-activated transcription factor that plays a key role in regulating the expression of genes encoding drug-metabolizing enzymes and drug transporters. It was subsequently found to potentiate various biologic effects associated with pharmacologic and toxicologic consequences—not only the metabolism and clearance of endobiotics and xenobiotics, including various therapeutic drugs and environmental endocrine disruptors, but also cancer pathogenesis (Timsit and Negishi, 2007; Staudinger and Lichten, 2008; Ihnnah et al., 2011; Pondugula and Mani, 2013) and hepatic energy metabolism including gluconeogenesis, the β-oxidation of fatty acids, and lipogenesis (Konno et al., 2008). PXR is activated by the binding of endogenous and exogenous xenobiotics to its ligand-binding domain and is then translocated to the nucleus in which it activates transcription by binding to the xenobiotic-response element in the promoter region of the target gene with RXR (Timsit and Negishi, 2007). However, less is known about the molecular mechanism underlying the ligand-dependent nuclear translocation of PXR, whereas the molecular mechanism responsible for the phenobarbital-induced nuclear translocation of CAR by the inhibition of epidermal growth factor receptors signaling and phosphorylation/dephosphorylation of the threonine-38 of hCAR has already been reported (Mutoh et al., 2013).

Previous studies indicated that the phosphomimetic mutation at the threonine-290 of hPXR abolished nuclear translocation and, thus, transactivation activity after ligand-independent stimulation with roscovitine; however, these studies rested on showing the protein kinase involved in the phosphorylation of threonine-290 and the molecular mechanism underlying nuclear translocation by ligand-dependent stimulation. Our study has demonstrated that threonine-290 is phosphorylated when hPXR was retained in the cytoplasm and its dephosphorylation facilitated the nuclear translocation of hPXR in response to ligand-dependent (rifampicin) and ligand-independent (roscovitine) stimulations (Figs. 1, 2, and 5).

Post-translational modifications in hPXR by phosphorylation have been shown to alter its function. Lin et al. (2008) and Sugatani et al. (2012) showed that CDK2 directly phosphorylated the serine-350 of hPXR and negatively affected its activity. Our previous studies (Sugatani et al., 2012) revealed that, whereas the phosphomimetic mutation at serine-350 of hPXR strongly impaired the interaction between the mutant hPXR with RXR and its coactivator SRC-2, the S350D mutant protein was able to translocate to the nucleus after the roscovitine treatment, similar to the wild-type protein. Furthermore, Pondugula et al. (2009) reported that a phosphomimetic mutation at the threonine-57 of hPXR abolished binding ability to the CYP3A4 promoter, and the 70-kDa form of ribosomal protein S6 kinase directly phosphorylated the threonine-57 of hPXR to suppress transactivation activity. Most of the T57D mutant protein can be translocated to the cell nucleus (Supplemental Fig. 1).

Using overexpression studies with Flag-tagged and YFP-tagged hPXR, we further identified threonine-290 as the phosphorylation site of hPXR such that rifampicin was no longer capable of translocating into the nucleus not only in cultured cells such as HepG2, HuH6, and SW-480 cells, but also in the mouse liver (Figs. 1 to 4). Because the hPXR T290A mutant was retained in the cytoplasm of nontreated cells, similar to the wild-type hPXR, and translocated into the nucleus only after the treatment with rifampicin and roscovitine, the dephosphorylation of threonine-290 is required but not sufficient for regulating the nuclear translocation of hPXR (Figs. 1 to 2). The mutation of threonine-290 to Asp induced hPXR to form a complex with Hsp90 and DNAJC7; however, the hPXR T290A mutant formed a complex with Hsp90 and DNAJC7 to some extent, similar to the wild-type hPXR, which suggests that other factors may contribute to the regulation of nuclear translocation.

While phosphorylated hPXR T290A mutant proteins were detected on Phos-tag gels, the levels of unphosphorylated proteins were not increased further after the treatment with rifampicin (Figs. 5 and 7). Previous studies (Lichti-Kaiser et al., 2009a; Sugatani et al., 2012) demonstrated that although phosphomimetic mutations at the threonine-57, threonine-390, serine-350, and threonine-408 of hPXR markedly suppressed the activity of hPXR, those of other serines/threonines slightly affected its activity; a phosphomimetic mutation at serine-130, which is involved in another CaMKII phosphorylation consensus sequence, did not inhibit basal translocation activity. We further demonstrated that hPXR phosphorylated at T408 may be degraded by proteasomes because the amount of the hPXR T408D mutant protein is lower than that of the wild-type protein. Furthermore, the S350D mutant protein was able to translocate to the nucleus after the roscovitine treatment, similar to the wild-type protein. Moreover, P旭atani et al. (2009) reported that a phosphomimetic mutation at threonine-38 of hCAR markedly suppressed the activity of hCAR, those of other serines/threonines slightly affected its activity; a phosphomimetic mutation at serine-130, which is involved in another CaMKII phosphorylation consensus sequence, did not inhibit basal translocation activity. We further demonstrated that hPXR phosphorylated at T408 may be degraded by proteasomes because the amount of the hPXR T408D mutant protein is lower than that of the wild-type protein. Furthermore, the S350D mutant protein was able to translocate to the nucleus after the roscovitine treatment, similar to the wild-type protein. Moreover, we have shown that phosphorylation of CaMKII in vivo. However, our results indicate a direct interplay between hPXR and CaMKII; the interaction between hPXR and active CaMKII, which was phosphorylated at threonine-286, was detected in nontreated cells (Fig. 9). The treatment with KN93 and anti-CaMKII siRNA also increased the dephosphorylated levels of the
hPXR wild-type protein (Figs. 5–7). It appears likely that CaMKII may play a key role in controlling the cellular localization of hPXR.

Although okadaic acid at a low nanomolar concentration preferentially suppressed PP2A, it suppressed the induction of UGT1A1 and CYP3A4 mRNAs by rifampicin and roscovitine in HepG2 cells only at a high concentration (100 nM), which suggests that it inhibited other serine/threonine phosphatasases such as PP1 (Fig. 10). Furthermore, the suppressed expression of PP1 by anti-PP1 siRNA led to the repressed expression of CYP3A4 mRNA in HepG2 cells treated with rifampicin, whereas the suppression of PP2 using anti-PP2 siRNA did not (Fig. 10). These results suggest that PP1 may be involved in activating hPXR in HepG2 cells stimulated with rifampicin.

Therefore, we investigated whether the YFP-hPXR-Hsp90 complex contained PP1 by precipitating the YFP-hPXR fusion protein with the anti-GFP antibody from the cytoplasmic fractions of cells treated with or without rifampicin or roscovitine. The PP1β subunit was markedly coprecipitated from the cytoplasmic fractions of ripsampicin- and roscovitine-stimulated cells (Fig. 11 and Supplementary Fig. 2). Our study has demonstrated for the first time that PP1 recruits the hPXR-Hsp90 complex when cells are stimulated to induce target genes in ligand-dependent and ligand-independent manners. Yoshinari et al. (2003) demonstrated that the cytoplasmic CAR-Hsp90 complex recruited PP2A in response to phenobarbital or phenobarbital-type inducers. The protein phosphatase involved in hPXR nuclear translocation was identified as PP1, which was different from PP2A involved in CAR nuclear translocation. However, our present results, in which the recruitment of serine/threonine protein phosphatase to the hPXR-Hsp90 complex upon ligand-dependent and ligand-independent induction was the regulatory step of its nuclear translocation, are consistent with previous findings on CAR nuclear translocation (Yoshinari et al., 2003).

PP1 is a key regulator of CaMKII, which functions as a serine/threonine kinase by autophosphorylation at its threonine-286 through the inhibition of PP1, leading to the regulation of synaptic strength in forebrain postsynaptic density (Strack et al., 1997a; Blitzer et al., 1998; Baucum II et al., 2012) and mediates PI3K activation in bovine sperm capacitation (Rotfeld et al., 2014). Furthermore, the dephosphorylation of CaMKII in postsynaptic density was previously shown to be catalyzed by PP1 (Shield et al., 1985; Dosemeci and Reece, 1993; Strack et al., 1997b). Therefore, as a schematic model of the signaling pathway for hPXR nuclear translocation (Fig. 13), we suggest 1) that active CaMKII phosphorylates the threonine-290 of hPXR, which is retained in the cytoplasm, and KN93 inhibits CaMKII autophosphorylation and hPXR phosphorylation by CaMKII, and 2) that PP1 recruits the hPXR-Hsp90 complex upon the rifampicin and roscovitine stimulations and dephosphorylates hPXR. It remains unclear whether the CaMKII/PP1 signaling pathway regulates hPXR nuclear translocation in vivo and also whether a signaling pathway exists that translocates dephosphorylated hPXR to the nucleus. We are now examining the phosphorylation/dephosphorylation of hPXR by CaMKII/PP1 in vivo, the molecular mechanism by which CaMKII and PP1 are regulated during the cytoplasmic retention and nuclear translocation of hPXR, as well as factors interacting with dephosphorylating hPXR, which may provide a novel insight into hPXR nuclear translocation signaling.

Fig. 13. Schematic model of the signaling pathway for hPXR nuclear translocation; regulation through phosphorylation/dephosphorylation at the threonine-290 of hPXR by CaMKII and PP1.


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