Threonine-408 Regulates the Stability of Human Pregnane X Receptor through Its Phosphorylation and the CHIP/Chaperone-Autophagy Pathway

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ABSTRACT

The human pregnane X receptor (hPXR) is a xenobiotic-sensing nuclear receptor that transcriptionally regulates drug metabolism-related genes. The aim of the present study was to elucidate the mechanism by which hPXR is regulated through threonine-408. A phosphomimetic mutation at threonine-408 (T408D) reduced the transcriptional activity of hPXR and its protein stability in HepG2 and SW480 cells in vitro and mouse livers in vivo. Proteasome inhibitors (calpain inhibitor I and MG132) and Hsp90 inhibitor geldanamycin, but not Hsp70 inhibitor pifithrin-μ, increased wild-type (WT) hPXR in the nucleus. The translocation of the T408D mutant to the nucleus was significantly reduced even in the presence of proteasome inhibitors, whereas the complex of yellow fluorescent protein (YFP)-hPXR T408D mutant with heat shock cognate protein 70/heat shock protein 70 and carboxy terminus Hsp70-interacting protein (CHIP; E3 ligase) was similar to that of the WT in the cytoplasm. Treatment with rifampicin, suggesting the contribution of Hsp70 and CHIP to the transactivation of hPXR. Autophagy inhibitor 3-methyladenine accumulated YFP-hPXR T408D mutant more efficiently than the WT in the presence of proteasome inhibitor lactacystin, and the T408D mutant colocalized with the autophagy markers, microtubule-associated protein 1 light chain 3 and p62, which were contained in the autophagic cargo. Lysosomal inhibitor chloroquine caused the marked accumulation of the T408D mutant in the cytoplasm. Protein kinase C (PKC) directly phosphorylated the threonine-408 of hPXR. These results suggest that hPXR is regulated through its phosphorylation at threonine-408 by PKC, CHIP/chaperone–dependent stability check, and autophagic degradation pathway.

Introduction

The pregnane X receptor (PXR) is a member of the nuclear receptor superfamily and is recognized as a xenobiotic sensor that regulates the transcriptional expression of phase I, II, and III metabolic enzymes and transporters involved in the metabolism and elimination of potentially toxic substances (reviewed in Timsit and Negishi, 2007; Staadinger and Lichti, 2008). PXR also plays important roles in many biologic events such as hepatic energy metabolism, immune/inflammatory responses, cell proliferation, bone homeostasis, and the pathogenesis of inflammatory bowel disease and tumor development (reviewed in Konno et al., 2008; Ihnann et al., 2011; Zhuo et al., 2014; Rathod et al., 2014; Ma et al., 2015). A previous study demonstrated that PXR was sequestered in the cytoplasm through the formation of a complex with heat shock protein (Hsp) 90 (Squires et al., 2004). Upon the binding of PXR ligands to the ligand binding domain, it translocates to the nucleus, in which it forms a heterodimer with retinoid X receptor and binds to the xenobiotic-response element of the target gene, resulting in transcriptional activation (Kawana et al., 2003; Squires et al., 2004).

Post-translational modifications to the human PXR (hPXR) by phosphorylation and through small ubiquitin-related modifier- and ubiquitin-signaling pathways have been shown to influence the functions of hPXR by modulating intracellular localization and nuclear activation (Lichii-Kaiser et al., 2009; Pondugula et al., 2009; Staadinger et al., 2011; Sugatani et al., 2012, 2014; Smutny et al., 2013; Elias et al., 2014; Cui et al., 2015). Cyclin-dependent kinase 2 has been shown to phosphorylate hPXR at serine-350 to suppress the binding with retinoid X receptor by maintaining the acetylation of the hPXR gene, resulting in transcriptional activation (Kawana et al., 2003; Squires et al., 2004). We previously reported that phosphomorphic mutations in hPXR at threonine-290 and threonine-408 suppressed the translocation of hPXR protein to the nucleus even after stimulation with roscovitine, resulting in reduced hPXR activities (Sugatani et al., 2012).

ABBREVIATIONS: 3-MA, 3-methyladenine; CAR, constitutive androstane receptor; CHIP, carboxy terminus Hsp70-interacting protein; DAPI, 4,6-diamidino-2-phenylindole dihydrochloride; DYRK2, dual-specificity tyrosine-(Y)-phosphorylation–regulated kinase 2; GFP, green fluorescent protein; GR, glucocorticoid receptor; HOP, stress-induced phosphoprotein 1; hPXR, human pregnane X receptor; Hsc70, heat shock cognate protein 70; Hsp, heat shock protein; LC3, microtubule-associated protein 1 light chain 3; MBP, maltose binding protein; PKC, protein kinase C; RBCK1, Ring-B-box-coiled-coil protein interacting with protein kinase C-1; siRNA, small-interfering RNA; UBR5, ubiquitin protein ligase E3 component n-recognin 5; UGT, UDP-glucuronosyltransferase; YFP, yellow fluorescent protein.

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Furthermore, we revealed that Ca\(^{2+}\)/calmodulin-dependent protein kinase II (CaMKII) directly phosphorylated hPXR at threonine-290 for its retention in the cytoplasm, and protein phosphatase 1 was recruited to the hPXR-Hsp90 complex and dephosphorylated hPXR in ligand-stimulated HepG2 cells (Sugatani et al., 2014). Since the stability of hPXR is known to be regulated through a proteasome degradation pathway (Rana et al., 2013; Ong et al., 2014) and the hPXR T408D mutant accumulates in the cytoplasm in the presence of proteasome inhibitor MG132 (Sugatani et al., 2012), the phosphorylation of hPXR at threonine-408 appears to be involved in the regulation of its stability. However, the molecular mechanisms regulating the stability and function of the T408D mutant have not been elucidated in detail (Sugatani et al., 2012).

Most cellular proteins in eukaryotic cells are degraded through two major pathways: the ubiquitin-proteasomal pathway and an autophagic process delivering cytoplasmic proteins and components to the lysosome (reviewed in Abounit et al., 2012; Lilienbaum, 2013). Heat shock proteins Hsc70/Hsp70 assist in the refolding of cytoplasmic proteins with cochaperone proteins such as the carboxy terminus Hsp70-interacting protein (CHIP) (Kampinga et al., 2003). Chaperone-dependent E3 ligase CHIP leads to the ubiquitination of misfolded proteins, which is facilitated by chaperone proteins to proteolytic degradation through an autophagic process (Kampinga et al., 2003; Morishima et al., 2008). To elucidate the role of phosphorylation at threonine-408, we herein systematically characterized and compared the expression of the hPXR T408D mutant and its interactions with chaperone proteins in the cytoplasm and nucleus with the wild-type (WT) in the presence and absence of proteasome inhibitors and an Hsp90 inhibitor, which induced chaperone protein Hsp70 and caused autophagy. We revealed that 1) the hPXR T408D mutant was expressed to the same extent as the WT, but was cleared more rapidly from the cytoplasm, as did the WT in HepG2 cells. Furthermore, the autophagy inhibitor, 3-methyladenine (3-MA) resulted in an increase in autophagy markers such as ATG12 and LC3 and p62, while the WT and the hPXR T408D mutant formed complexes with chaperone proteins Hsp90–Hsc70/Hsp70, as well as cochaperone proteins such as CHIP, stress-inducible phosphoprotein 1 (HOP), and p23 in the cytoplasm, as did the WT in HepG2 cells. Moreover, the CHIP/chaperone-autophagy system in the cytoplasm.

**TABLE 1**

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**Materials and Methods**

**Materials.** MG132, calpain inhibitor I, lactacystin, and geldanamycin were purchased from Calbiochem (Darmstadt, Germany). Rifampicin, chloroquine diphosphate, and 3-MA were obtained from Sigma-Aldrich (St. Louis, MO). Pifithrin-μ was from Cayman Chemical Company (Ann Arbor, MI), adenosine-5′-triphosphate (ATP) 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, NC). Mutations were induced in hPXR with a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer’s instructions, and expression vectors were prepared as shown in our previous study (Sugatani et al., 2012, 2014).

**Cell Culture Conditions, Transfection, and Luciferase Assays.** Human liver-derived cells (HepG2 cells, the RIKEN BioResource Center, Ibaraki, Japan) were seeded on 6-well plates at 2 × 105 cells (2 ml) in Dubelco’s modified Eagle’s medium supplemented with 10% fetal calf serum (HyClone, GE Healthcare Life Sciences, Logan, UT) and antibiotics (100 μg of streptomycin and 10 IU of penicillin/ml) at 37°C in the presence of 5% CO2, unless otherwise stated. Twenty-four hours later, HepG2 cells were transfected with 0.02 μg of luciferase reporter constructs including the distal (-3570/-3180) and proximal (-165/-1) regions (Sugatani et al., 2001, 2008) (0.2 μg) and expression vectors (pCR3 vector (Invitrogen/Thermo Fisher Scientific, Sunnyvale, CA) (0.2 μg), pCR3-hPXR WT (0.2 μg), pCR3-hPXR T408A (0.2 μg), pCR3-hPXR T408D (0.2 μg), and pRL-SV40 (Promega, Madison, WI) (0.2 μg)) using HilyMax reagent (Dojindo Laboratories, Kamakura, Japan) according to the manufacturer’s instructions. The medium (0.5 ml per well) was replaced after 12 hours with the same medium. Cells were subsequently treated for 24 hours with rifampicin (5 × 10⁻⁶ M) or 400 × concentrated stocks in dimethylsulfoxide; controls received an equivalent volume of dimethylsulfoxide. The total amount of DNA transfected was held constant in each transfection by using the corresponding empty vector. Transfected cells were washed once in phosphate-buffered saline and harvested in 1× passive lysis buffer, and luciferase activity was measured simultaneously with the Dual-Luciferase reporter assay system according to the manufacturer’s instructions (Promega); firefly luciferase values were normalized to Renilla values for each sample.

**Real-Time Quantitative PCR.** Since PXR is a key transcriptional regulator of CYP3A4 (Lehmann et al., 1998) and human UGT1A1 (Sugatani et al., 2004, 2005), we used CYP3A4 and UGT1A1 as biomarkers of the activation of hPXR. HepG2 cells and SW480 human colon cancer cells (American Type Culture Collection) (2 × 10⁵ cells) seeded on 6-well plates and cultured for 24 hours in Dulbecco’s modified Eagle’s medium (HepG2 cells) and RPMI 1640 medium (SW480 cells), respectively, supplemented with 10% fetal calf serum (Hyclone) and antibiotics (100 μg of streptomycin and 10 IU of penicillin/ml) were transfected with expression vectors (pCR3 (0.2 μg), pCR3-hPXR (0.2 μg), or pCR3-hPXR mutant (0.2 μg)) using TransIT-LT1 (Mirus Bio, Madison, WI) according to the manufacturer’s instructions. Cells were given fresh medium.
24 hours after being transfected and were then retransfected with the expression vectors using the same protocol as that for the first transfection. Ten minutes after the second transfection, cells were cultured with rifampicin (5 × 10⁻⁶ M) or vehicle in the presence or absence of various inhibitors for an additional 24 hours, unless otherwise stated. Total RNA was extracted using TRIzol reagent from Invitrogen/Thermo Fisher Scientific, and cDNA synthesized from 75 ng of total RNA by reverse transcription with a Prime Script reverse transcription reagent kit (Takara Bio, Otsu, Japan) was subjected to a quantitative real-time PCR as described previously (Sugatani et al., 2012) using SYBR Premix Ex Taq reagent (Takara Bio) according to the manufacturer’s specifications for UGT1A1 and CYP3A4 (Sugatani et al., 2010), Hsp90, Hsp70, Hsc70, Hop, RBCK1, and UBR5 (Takara Bio), and CHIP (Kajiro et al., 2009). All primers used for real-time PCR were listed in Table 1.

Expression of the Flag-Tagged hPXR WT and T408D Mutant Proteins in Mouse Livers In Vivo. To investigate the stability of the hPXR WT and T408D mutant, plasmids [pCMV-DYKDDDDK-hPXR WT (1 μg) or the pCMV-DYKDDDDK-hPXR T408D mutant (1 μg)] were injected into the tail veins of male imprinting-control-region (ICR) mice (4 weeks old) using the TransIT-QR Hydrodynamic Delivery Starter Kit (Mirus Bio) according to the manufacturer’s instructions. Twelve and 24 hours later, mice were rapidly excised and weighed. Whole livers were 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 14,000g for 10 minutes. Protein concentrations were determined using a DC protein assay (Bio-Rad, Hercules, CA).

Western Blot Analysis. HepG2 cells (2 × 10⁶ cells) seeded on 75-cm² flasks and cultured for 24 hours were transfected with expression vectors [pCR3-hPXR WT (10 μg), the pCR3-hPXR T408D mutant (10 μg), pCMV-DYKDDDDK-hPXR WT (10 μg), the pCMV-DYKDDDDK-hPXR T408D mutant (10 μg), pEYPF-hPXR WT (10 μg), or the pEYPF-hPXR T408D mutant (10 μg)] according to the TransIT-LT1 (Mirus Bio) manufacturer’s instructions. Cells that were given fresh medium 24 hours after transfection, were further transfected with the expression vectors, and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle for an additional 24 hours unless stated otherwise. Treated and untreated cells were washed three 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, 1mM 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 14,000g for 10 minutes. Nuclear extracts and cytoplasmic fractions were prepared using a nuclear extract kit (Active Motif, Carlsbad, CA). Protein concentrations were determined using the DC protein assay (Bio-Rad). Cytoplasmic fractions, nuclear extracts, cell lysates, or liver lysates (20 μg) were resolved by electrophoresis on an SDS 5–20% polyacrylamide gel (ATTO Corporation, Tokyo, Japan), and electroblotted onto a polyvinylidene difluoride membrane (Merck Millipore, Darmstadt, Germany). 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-DDDDK (Flag) tag (M1853-3L), anti-green fluorescent protein (GFP) (598), anti-LC3 (M152-3), and anti-p62/SQSTM1 (M162-3) from Medical Biologic Laboratories (Nagoya, Japan), anti-Hsp90 (#4874) from Cell Signaling Technology (Danvers, MA), anti-Histone H1 (ab120927), anti-Hsp70 (ab2787), anti-HOP (ab56873), anti-CHIP (ab109103), and anti-p23 (ab2814) from Abcam (Cambridge, England), anti-Hsc70 (SCM-151A/B) from StressMarq Bioscience (Victoria, BC, Canada), anti-ubiquitin (MAB701) from R&D Systems (Minneapolis, MN), anti-PXR (AB10302) from Merck Millipore, and anti-α-tubulin (CP06) from Calbiochem/Merck Millipore. 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 Immuno Research)] and visualized with an enhanced chemiluminescence system (GE Healthcare, Little Chalfont, England).

Coimmunoprecipitation. HepG2 cells (2 × 10⁶ cells) seeded on 75-cm² flasks and cultured for 24 hours were transfected with expression vectors [pEYPF-hPXR WT (10 μg) or the pEYPF-hPXR T408D mutant (10 μg)] using TransIT-LT1 according to the manufacturer’s instructions. Cells were given fresh medium 24 hours after transfection, and further transfected with the expression vectors, and cultured with rifampicin (5 × 10⁻⁶ M) or vehicle for an additional 24 hours. The cytoplasmic (530 μg) and nuclear (200 μg) proteins, unless otherwise stated, were precleared with protein G agarose beads and rabbit IgG (Santa Cruz Biotechnology, Dallas, TX) for 30 minutes at 4°C. Yellow fluorescent protein (YFP)-tagged proteins were immunoprecipitated using an anti-GFP antibody combined with protein G agarose beads. The agarose beads

Fig. 1. Transactivation capacity of the hPXR T408D mutant in HepG2 and SW480 cells (A–C), and its protein stability in vitro in HepG2 cells (D) and in vivo in mouse livers (E). (A–C) HepG2 and SW480 cells cultured for 24 hours were transfected with expression vectors encoding the hPXR WT or T408D mutant. The medium was replaced 24 hours after transfection, and cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle (DMSO) for an additional 24 hours. UGT1A1 reporter activity assessed by the expression of a reporter gene encoding firefly luciferase (A), and CYP3A4 mRNA levels in HepG2 (B) and SW480 (C) cells treated with rifampicin or vehicle were expressed by taking the control value obtained from hPXR WT-expressing and vehicle-treated cells as one. Data presented are the average ± S.E. of three experiments. **P < 0.01; ***P < 0.001 versus the hPXR WT in cells treated without rifampicin; *P < 0.05; **P < 0.01; ***P < 0.001 versus the hPXR WT in cells stimulated with rifampicin. (D, E) A Western blot analysis of the Flag-tagged hPXR WT and T408D mutant proteins in HepG2 cells after the in situ DNA injection (D) and in the mouse livers 12 hours and 24 hours after the in situ DNA injection (E). HepG2 cells cultured for 33 hours were transfected with expression plasmids for hPXR. The medium was replaced 24 hours after transfection, and cells were cultured with MG132 (1 × 10⁻⁶ M) or vehicle (DMSO) for an additional 15 hours. Protein levels in cells before the treatment with MG132 for an additional 15 hours. Protein levels in cells before the treatment with MG132 were taken as the level at time 0. Flag-hPXR proteins in HepG2 cell lysates (D) or in liver lysates (E) were determined by Western blotting with the indicated antibodies [anti-Flag and anti-α-tubulin (loading control antibodies)]. (E) Band intensities were measured with ImageJ software, and each quantified value of the Flag-hPXR bands was corrected for that of α-tubulin. Relative levels are expressed as the protein levels obtained from mouse livers expressing Flag-hPXR WT 12 hours after the in situ DNA injection as one. Data presented are the average ± S.E. of three to five experiments. *P < 0.05 versus mouse livers expressing Flag-hPXR WT 24 hours after the in situ DNA injection. Western blotting were performed twice with similar results, and representative data are shown. WT, wild-type hPXR; T408A, T408A-mutated hPXR; T408D, T408D-mutated hPXR.
then cultured with rifampicin (5 μM) or vehicle for an additional 24 hours, unless stated otherwise. Treated and untreated cells were washed three 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 14,000g for 10 minutes. Total RNA was extracted using TRIZOL reagent from Invitrogen/Thermo Fisher Scientific, and cDNA synthesized from 75 ng of total RNA was subjected to real-time quantitative PCR as described previously (Sugatani et al., 2012).

In Vitro Kinase Assays. The maltose-binding protein (MBP)-hPXR WT and T408D mutant fusion proteins were expressed in NEB Express Competent E. coli (High Efficiency) and purified using amylose resin according to the manufacturer’s protocols (New England BioLabs, Boston, MA). Recombinant hPXR prepared by the cleavage of MBP-hPXR with Factor Xa, the recombinant His-tagged hPXR ligand-binding domain (138–434) (Sigma-Aldrich), or recombinant Hsc70 (StressMarq Biosciences) was incubated with Ub-His5 (UBE2D3, E2 ubiquitin-conjugating enzyme; Abcam) and CHIP (E3 ubiquitin protein ligase; R&D Systems) at 37°C for 30 minutes with an Alexa Fluor 555-conjugated anti-mouse IgG (H + L) antibody (Thermo Fisher Scientific). Coverslips were mounted with Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA) with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories) to visualize nuclei, and images were acquired with a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

In Vitro Ubiquitination Assays. The MBP-hPXR WT and T408D mutant fusion proteins were expressed in NEB Express Competent E. coli (High Efficiency) and cultured for 24 hours with rifampicin (5 μM) or vehicle for an additional 24 hours, unless stated otherwise. Cells were further transfected with expression vectors [the pCR3-hPXR WT, pCR3-hPXR T408D mutant, pCMV-DYKDDDDK-hPXR WT, or pCMV-DYKDDDDK-hPXR T408D mutant] 12 hours after siRNA transfection, and 12 hours after the second transfection were cultured with rifampicin (5 × 10⁻⁶ M) or vehicle for an additional 24 hours, unless stated otherwise. Treated and untreated cells were washed three 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 14,000g for 10 minutes. Total RNA was extracted using TRIZOL reagent from Invitrogen/Thermo Fisher Scientific, and cDNA synthesized from 75 ng of total RNA was subjected to real-time quantitative PCR as described previously (Sugatani et al., 2012).

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Colocalization of the Fluorescent Protein–Tagged hPXR WT or T408D Mutant and Autophagy Markers P62 and LC3 in HepG2 Cells. To detect the colocalization of the hPXR WT or T408D mutant and the autophagy markers p62 and LC3, HepG2 cells (3.5 × 10⁴ /well) cultured on four-well glass slides (Laboratory-TekII Chamber Slide, cat. no. 154526; Thermofisher Scientific) for 24 hours were transfected with expression vectors [the pEYFP-hPXR WT (0.5 μg) or pEYFP-hPXR T408D mutant (0.5 μg)] using TransIT-LT1 according to the manufacturer’s instructions. Twelve hours after transfection, cells were incubated with 3-methyladenine (5 mM) for 12 hours, and then further cultured with rifampicin (5 × 10⁻⁶ M) or vehicle in the presence or absence of lactacystin (5 μM) for an additional 24 hours. Cells were washed with methanol and incubated with the anti-LC3 (1:250) or anti-p62 (1:250) antibody at room temperature for 30 minutes. These cells were counterstained at room temperature for 30 minutes with an Alexa Fluor 555-conjugated anti-mouse IgG (H + L) antibody (1:1000) (Thermo Fisher Scientific). Coverslips were mounted with Vectashield antifade mounting medium (Vector Laboratories Inc., Burlingame, CA) with 4,6-diamidino-2-phenylindole dihydrochloride (DAPI; Vector Laboratories) to visualize nuclei, and images were acquired with a Zeiss LSM510 confocal microscope (Carl Zeiss, Oberkochen, Germany).

In Vitro Kinase Assays. The MBP-hPXR WT and T408A mutant fusion proteins were expressed in NEB Express Competent E. coli (High Efficiency) and cultured with rifampicin (5 μM) or vehicle for an additional 24 hours, unless stated otherwise. Cells were further transfected with expression vectors [the pCR3-hPXR WT, pCR3-hPXR T408D mutant, pCMV-DYKDDDDK-hPXR WT, or pCMV-DYKDDDDK-hPXR T408D mutant] 12 hours after siRNA transfection, and 12 hours after the second transfection were cultured with rifampicin (5 × 10⁻⁶ M) or vehicle for an additional 24 hours, unless stated otherwise. Treated and untreated cells were washed three 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 14,000g for 10 minutes. Total RNA was extracted using TRIZOL reagent from Invitrogen/Thermo Fisher Scientific, and cDNA synthesized from 75 ng of total RNA was subjected to real-time quantitative PCR as described previously (Sugatani et al., 2012).
and purified using amylose resin according to the manufacturer’s protocol (New England Biolabs, Boston, MA). Kinase assays for protein kinase C (PKC; Promega) and DYRK2 (SignalChem Lifesciences, Richmond, BC, Canada) were performed according to the manufacturer’s specifications. PKC consisted primarily of the α, β, and γ isoforms with lesser amounts of the δ and ε isoforms (Walton et al., 1987). Briefly, kinase reactions were performed in 30 µl of the kinase buffer (for PKC, 20 mM HEPES (pH 7.4), 3.4 mM CaCl₂, and 10 mM MgCl₂; for DYRK2, 25 mM MOPS (pH 7.2), 12.5 mM β-glycerophosphate, 25 mM MgCl₂, 5 mM EGTA, 2 mM EDTA, and 0.25 mM dithiothreitol) containing 0.75–4.5 µg of the substrate protein, 100 µM ATP, and 0.12 MBq of [γ-32P]ATP at 30°C for 10 and 20 minutes, unless otherwise stated. Reactions were stopped by adding the same volume of 2× 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 5–20% gradient gel (ATTO Corporation) after heat denaturation. Phosphorylation of the substrate was visualized by autoradiography.

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

Results

Phosphomimetic Mutation of hPXR at T408 Reduced the Induction of CYP3A4 mRNA by Rifampicin. We previously reported that the phosphomimetic mutation of hPXR at T408 attenuated the induction of UGT1A1 mRNA expression by roscovitine and rifampicin in HepG2 cells (Sugatani et al., 2012). The present study also revealed that the phosphomimetic T408D mutation attenuated UGT1A1 reporter activity by rifampicin, whereas the phosphodeficient T408A mutation exhibited similar activity to that of the WT (Fig. 1A). Furthermore, the T408D mutation reduced the induction of CYP3A4 mRNA by rifampicin not only in HepG2 but also in SW480 cells (Fig. 1, B and C). We then determined whether the phosphomimetic mutation of hPXR at T408 altered the expression of hPXR in HepG2 cells in vitro.

Fig. 3. Immunoprecipitation and Western blot analyses of the nucleocytoplasmic distribution of the hPXR WT and T408D mutant and their interactions with chaperone and co-chaperone proteins in proteasome inhibitor–treated HepG2 cells. HepG2 cells cultured for 24 hours were transfected with expression vectors encoding the YFP-hPXR WT or T408D mutant. The medium was replaced 24 hours after transfection, and cells were retransfected and then cultured with rifampicin (5×10⁻⁶ M) or vehicle (DMSO) in the presence or absence of calpain inhibitor I (1×10⁻³ M), MG132 (1×10⁻⁶ M), or vehicle (DMSO, control) for an additional 24 hours. Cytoplasmic, 530 µg (A) and nuclear, 200 µg (B) proteins prepared from these cells and a blank sample (vehicle) were precipitated with anti-GFP antibody. The resulting bound proteins were subjected to a Western blot analysis with the indicated antibodies. Cytoplasmic and nuclear YFP-tagged hPXR and chaperone proteins were determined by Western blotting using the indicated antibodies: anti-α-tubulin (loading control) and anti-histone H1 (loading control) antibodies. Experiments were performed three times with similar results, and representative data are shown. WT, wild-type hPXR; T408D, T408D-mutated hPXR.
and in mouse livers in vivo or reduced the stability of hPXR, resulting in impaired transcriptional activity. No significant change was observed in the protein levels of the hPXR WT or T408D mutant expressed 24 hours after transfection with expression plasmids for hPXR, and 39 hours after transfection the level of the T408D mutant protein was reduced in the absence of MG132 but increased to a level similar to that of the WT protein in the presence of MG132 (Fig. 1D). We also investigated the expression of the hPXR WT and T408D mutant proteins in mouse livers after the in situ WT- or T408D mutant-expressing plasmid DNA injection. Whereas no significant difference was observed in the levels of the hPXR WT and T408D mutant proteins expressed in mouse livers 12 hours after the in situ DNA injection, the levels of the T408D mutant protein were significantly lower than those of the WT protein 24 hours after the in situ DNA injection (Fig. 1E). These results suggested that the impaired function of the phosphomimetic mutant was not caused by its reduced expression, and also that phosphorylation at T408 reduced the protein stability of hPXR, thereby impairing its function.

**Effects of Proteasome Inhibitors on the Nucleocytoplasmic Distribution of the hPXR WT and T408D Mutant and Their Interactions with Chaperone and Cochaperone Proteins.** Since hPXR stability was previously shown to be regulated via phosphorylation-facilitated ubiquitination through dual-specificity tyrosine-(Y)-phosphorylation–regulated kinase 2 (DYRK2) and

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**Fig. 4.** Effects of proteasome inhibitors on the nucleocytoplasmic distribution of the hPXR WT and T408D mutant (A, B) and their interactions with chaperone and cochaperone proteins (C-F). The band intensities shown in Fig. 3 were measured with ImageJ software. (A, B) Each quantified value of the YFP-hPXR bands was corrected for that of α-tubulin (A) or histone H1 (B), and relative levels are expressed by taking the values obtained from cells expressing the YFP-hPXR WT and treated without rifampicin as one. (C–F) Cytosolic YFP-PXR was immunoprecipitated using an anti-GFP antibody and other proteins bound to PXR were detected by Western blotting. Each quantified value of the cytosolic YFP-hPXR, Hsp90, Hsp70, Hsc70, and CHIP bands was corrected for that of IgG heavy chain (HC). Relative levels of the hPXR WT and T408D mutant (A, D), expression of chaperone and cochaperone mRNAs in cells expressing the hPXR WT (B, C), and nucleocytoplasmic distribution of the hPXR WT and T408D mutant (E) in HepG2 cells. HepG2 cells cultured for 24 hours were transfected with expression plasmids for hPXR. The medium was replaced 24 hours after transfection, and cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle (DMSO) in the presence of pifithrin-μ (1 × 10⁻⁶ M), geldanamycin (1 × 10⁻⁶ M), or vehicle (DMSO, control) for an additional 24 hours. (A–D) Messenger RNA levels in control cells treated without rifampicin were taken as one. Data presented are the average ± S.E. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 versus the hPXR WT in vehicle-treated control cells; #P < 0.05; ##P < 0.01; ###P < 0.001 versus the hPXR WT in vehicle-treated control cells; a, P < 0.05 versus the hPXR WT in vehicle-treated control cells; b, P < 0.05; bb, < 0.01 versus the hPXR WT in rifampicin-stimulated control cells; cc, P < 0.01 versus the hPXR T408D mutant in vehicle-treated control cells; d, P < 0.05 versus the hPXR WT in rifampicin-stimulated control cells; e, P < 0.05 versus the hPXR WT in vehicle-treated cells with calpain inhibitor I; f, P < 0.05 versus the hPXR WT in rifampicin-stimulated cells with calpain inhibitor I; g, P < 0.05 versus the hPXR WT in vehicle-treated cells with MG132; h, P < 0.05 versus the hPXR WT in rifampicin-stimulated cells with MG132. WT, wild-type hPXR; T408D, T408D-mutated hPXR.

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**Fig. 5.** Effects of geldanamycin (A–C) and pifithrin-μ (D, E) on the transactivation capacity of the hPXR WT and T408D mutant (A, D), expression of chaperone and cochaperone mRNAs in cells expressing the hPXR WT (B, C), and nucleocytoplasmic distribution of the hPXR WT and T408D mutant (E) in HepG2 cells. HepG2 cells cultured for 24 hours were transfected with expression plasmids for hPXR. The medium was replaced 24 hours after transfection, and cells were retransfected and then cultured with rifampicin (5 × 10⁻⁶ M) or vehicle (DMSO) in the presence of pifithrin-μ (1 × 10⁻⁶ M), geldanamycin (1 × 10⁻⁶ M), or vehicle (DMSO, control) for an additional 24 hours. (A–D) Messenger RNA levels in control cells treated without rifampicin were taken as one. Data presented are the average ± S.E. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 versus the hPXR WT in vehicle-treated control cells; #P < 0.05; ##P < 0.01; ###P < 0.001 versus the hPXR WT in vehicle-treated control cells; †P < 0.05 versus the hPXR WT in rifampicin-stimulated control cells; ‡P < 0.05 versus the hPXR WT in rifampicin-stimulated control cells; ‡‡P < 0.01 versus the hPXR WT in rifampicin-stimulated control cells; a, P < 0.001 versus the hPXR T408D mutant in rifampicin-stimulated control cells. (E) Cytosolic and nuclear YFP-tagged hPXR from cells treated with pifithrin-μ (1 × 10⁻⁵ M) or vehicle (DMSO, control) were determined by Western blotting using anti-GFP, anti-α-tubulin (loading control), and anti-histone H1 (loading control) antibodies. Experiments were performed twice with similar results, and representative data are shown. WT, wild-type hPXR; T408D, T408D-mutated hPXR.
ubiquitin/proteasome pathway.

**Effects of Hsp90 and Hsp70 Inhibitors on Transcriptional Activities and Nucleocytoplasmic Distribution of the T408D Mutant, and Its Complex Formation with Chaperone and Cochaperone Proteins in HepG2 Cells.** We investigated the mechanisms by which the inhibition of Hsp90 and Hsp70 influenced the transcriptional activity and cellular distribution of hPXR using the Hsp90 inhibitor geldanamycin and Hsp70 inhibitor pifithrin-μ. Geldanamycin at 1 μM increased the basic levels of CYP3A4 mRNA in nonstimulated cells, and pifithrin-μ at 10 μM reduced CYP3A4 mRNA levels in the hPXR WT and T408D mutant-expressing rifampicin-stimulated cells, whereas cell growth was suppressed in cells treated with geldanamycin at 1 μM but not in those treated with pifithrin-μ at 10 μM (Fig. 5 and Supplemental Fig. 1).

Geldanamycin induced the expression of Hsp90 and Hsp70 mRNAs, did not alter Hsc70 or HOP mRNA levels, and reduced CHIP mRNA levels (Fig. 5 and Supplemental Fig. 2). Furthermore, it markedly increased Hsp70, hPXR WT, and T408D mutant protein levels in the cytoplasm, which then appeared to increase WT and T408D mutant protein levels in the nucleus, as well as CYP3A4 mRNA levels (Figs. 5 and 6). The immunoprecipitation assay revealed that geldanamycin induced complex formation by the hPXR WT and T408D mutant with Hsp70, Hsp70, CHIP, and HOP, but not p23 in the cytoplasm (Fig. 6). The binding of the hPXR WT and T408D mutant with Hsp90, CHIP, and p23 was not observed in the nucleus of geldanamycin-treated cells, the same as in the control and proteasome inhibitor-treated cells (Figs. 3 and 6). In contrast, pifithrin-μ at 10 μM accumulated the hPXR WT and T408D mutant proteins in the cytoplasm but not in the nucleus (Fig. 5). These results suggested that Hsp70 contributed to the folding of client proteins such as hPXR in the cytoplasm.

**CHIP Deletion Attenuated hPXR Transcriptional Activity in HepG2 Cells Treated with and without Lactacystin.** Since the knockdown of E3 ligases RBCK1 and UBR5 was previously reported to result in the accumulation of cellular hPXR and concomitant increases in the induction of hPXR target genes by rifampicin (Rana et al., 2013; Ong et al., 2014), we evaluated the effects of calpain inhibitor 1 and geldanamycin on the expression of CHIP, RBCK1, and UBR5 mRNAs. The inhibition of proteasome and Hsp90 did not markedly affect the expression of CHIP, RBCK1, or UBR5 mRNAs; the calpain inhibitor at 10 μM slightly increased CHIP and UBR5 mRNA levels but did not alter those of RBCK1 mRNA (Supplemental Fig. 2). Furthermore, geldanamycin at 1 μM reduced CHIP and RBCK1 mRNA levels but did not alter those of UBR5 mRNA (Supplemental Fig. 2). To examine the effects of CHIP knockdown, anti-CHIP siRNA was introduced into HepG2 cells and the hPXR WT and T408D mutant were then exogenously expressed in these cells. Transfection with anti-CHIP siRNA reduced CHIP mRNA levels to 0.29–0.33 fold of the control and CHIP protein levels in whole cells to 0.30–0.34 fold of the control, and also significantly attenuated the levels of CYP3A4 mRNA induced by rifampicin, demonstrating that CHIP may contribute to the transactivation of hPXR (Fig. 7). The
knockdown of CHIP led to the significant accumulation of the hPXR WT in cells treated without rifampicin in the presence of lactacystin (Fig. 7B). These results revealed that CHIP may contribute to the degradation of hPXR as well as its transactivation.

The Lysosome Inhibitor Chloroquine Increased the Stability of the hPXR T408D Mutant in the Cytoplasm. In an attempt to explore the hypothesis that the hPXR T408D mutant is degraded in lysosomes that fuse with an autophagic vacuole, we investigated the effects of chloroquine on the transcriptional activity and nucleocytoplasmatic distribution of the T408D mutant. Chloroquine at 20 μM, which did not affect cell growth, increased the induction of CYP3A4 mRNA by rifampicin and a concomitant increase was observed in hPXR WT protein levels in the nucleus (Supplemental Fig. 1 and Fig. 8). In contrast, the T408D mutant protein accumulated more efficiently than the WT in the cytoplasm but not in the nucleus in the presence of chloroquine, resulting in reduced transcriptional activities (Fig. 8). These results indicated that the T408D mutant was degraded in the lysosome.

The Complex of hPXR with Chaperone and Cochaperone Proteins Was Ubiquitinated in Cells Treated with or without Proteasome Inhibitors. To determine whether hPXR was directly ubiquitinated by E3 ubiquitin ligase CHIP, we assayed the in vitro ubiquitination of the hPXR WT by CHIP with the E2 ubiquitin-conjugating enzyme UbcH5c and compared with Hsc70, because Hsc70 is a major target of CHIP (Jiang et al., 2001). As shown in Fig. 9A, CHIP directly ubiquitinated Hsc70 but not the hPXR WT. In contrast, when immunoprecipitated proteins with the

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anti-GFP antibody for the cytoplasm of vehicle- and calpain inhibitor I-treated cells were immunoblotted for ubiquitin, a protein band near 73 kDa [band marked with two asterisks (***) in Fig. 9B] in the hPXR/chaperone complex and smeared bands were detected; however, the complex proteins were markedly smeared and polyubiquitinated for the cytoplasm of MG132-treated cells (Fig. 9B).

**Autophagic Cargo Contained hPXR.** Wu et al. (2010) reported that although 3-MA promoted an autophagic flux (resulting in an...
increase in autophagic markers) when treated under nutrient-rich conditions for a prolonged period of time, it was still capable of suppressing starvation-induced autophagy. Since inhibition of the proteasome has been shown to induce macroautophagy (Ding et al., 2007; Tang et al., 2014) and 3-MA blocks the maturation of the autophagophore into a closed autophagosome in the macroautophagic process (Abounit et al., 2012), we evaluated the effects of lactacystin and 3-MA on the cellular stability of hPXR and intracellular trafficking of the hPXR T408D mutant to the autophagic cargo to further explore our hypothesis that the hPXR T408D mutant is degraded through autophagy.

A Western blot analysis revealed that the treatment of the hPXR WT- or T408D mutant-expressing cells with lactacystin or 3-MA alone accumulated not only cellular hPXR WT but also the T408D mutant (Fig. 10). The hPXR T408D mutant accumulated more efficiently than the WT in cells treated with lactacystin in the presence of 3-MA. The autophagic markers LC3 and p62 markedly increased in cells treated with 3-MA and/or lactacystin in full medium. To determine whether the autophagic cargo contained hPXR, we examined the colocalization of the hPXR WT and T408D mutant with LC3 using an anti-LC3 antibody to identify the autophagic structure. Many LC3 puncta contained the hPXR WT and the T408D mutant (Fig. 11). Similar results were obtained for the ubiquitinated substrate adaptor p62 in cells treated with lactacystin and/or 3-MA (Fig. 11 and Supplemental Fig. 3). These results suggested that LC3- and p62-positive autophagic cargo functioned to deliver hPXR to the lysosome.

hPXR Was Phosphorylated at Threonine-408 by PKC In Vitro.

In our previous study (Sugatani et al., 2014), we demonstrated that the mRNA levels of UGT1A1 induced by rifampicin or roscovitine were slightly higher in HepG2 cells simultaneously treated with Go6983 (PKC inhibitor) but were lower in HepG2 cells simultaneously treated with Rp-8-Br-cAMPS (PKA inhibitor). Since hPXR contained the minimal recognition motif S/T-X-R at amino acids 408–410, which may potentially be phosphorylated by PKC (Kang et al., 2012), and DYRK2 phosphorylates hPXR to facilitate its subsequent ubiquitination by UBR5 (Ong et al., 2014), we performed assays with PKC or DYRK2 as protein serine/threonine kinase, [γ-32P]ATP and engineered MBP fusion proteins containing hPXR. In vitro kinase assays revealed that hPXR was phosphorylated by PKC and DYRK2, as assessed by radiography (Fig. 12A). To identify the phosphorylated site(s), we investigated whether the hPXR T408A mutant protein was phosphorylated by these kinases and found that mutating the threonine-408 of hPXR to alanine led to significantly lower phosphorylation levels by PKC than by the hPXR WT (0.73 ± 0.08 of the WT; P < 0.05 versus the WT), whereas DYRK2 phosphorylated the hPXR T408A mutant more than the hPXR WT (1.26 ± 0.10 of the WT; P < 0.05 versus the WT) (Fig. 12). Taken together, these results indicated that the threonine-408 of hPXR in the ligand-binding domain was one of the PKC-specific phosphorylation sites.

### Discussion

The glucocorticoid receptor (GR), which is a ligand-dependent nuclear receptor, is known to be phosphorylated through cell- and tissue-specific signaling systems, leading to conformational changes, which in turn modulate its transcriptional responses and stability (reviewed in Galliher-Beckley and Cidlowski, 2009). Previous studies demonstrated that phosphomimetic mutations at the threonine-57, threonine-290, serine-350, and threonine-408 of hPXR suppressed hPXR activities (Lichti-Kaiser et al., 2009; Pondugula et al., 2009; Sugatani et al., 2012, 2014; Wang et al., 2015). Therefore, hPXR functions are also considered to be modulated through the phosphorylation of hPXR at specific amino acid residues as well as nuclear receptors such as constitutive active/androstane receptor (CAR) and GR (Galliher-Beckley and Cidlowski, 2009; Mutoh et al., 2013). We also previously reported that the phosphorylation of hPXR at threonine-408 may be associated with its degradation through proteasomal machinery because the hPXR T408D mutant markedly accumulated in the cytoplasm of cells treated with MG-132 at an extremely high 5 μM concentration (Sugatani et al., 2012). However, the results of the present study suggest the presence of a proteasome-independent degradation pathway in addition to the proteasome-dependent degradation pathway because the protein level of the T408D mutant was less than or similar to that of the WT in the cytoplasm and significantly lower than the WT in the nucleus, even in the presence of proteasome inhibitors (Figs. 1–4). Thus, we herein examined the molecular mechanisms regulating hPXR functions and stability, especially those underlying autophagy for its degradation.

The chaperone proteins Hsc70/Hsp70 assist in the correct protein folding of nascent or denatured client proteins for intracellular functions.
and, in contrast, work with a ubiquitin pathway for the proteolytic degradation of unstable or misfolded client proteins (Hayer-Hartl et al., 1996; Wickner et al., 1999; Meacham et al., 2001; Kampina et al., 2003). CHIP interacts with Hsp70/Hsp90, which results in the refolding of chaperone complexes with client proteins, and is also involved in the direct ubiquitination of client proteins such as GR as an E3 ligase for delivery to the proteasome (reviewed in McDonough and Patterson, 2003). HOP has been shown to assist in protein transfer in the Hsp70- and Hsp90-dependent protein-folding pathways (Yamamoto et al., 2014). Hsp90, which regulates transcription by maintaining transcription factors in the cytoplasm, is known to interact with p23 and CHIP through different sites, and CHIP interacts with the carboxy-terminal domain of Hsc70 (reviewed in McDonough and Patterson, 2003). In addition to GR and CAR, hPXR has been identified as an Hsp70 client substrate (Sanchez et al., 1985; Honkakoski et al., 1998; Squires et al., 2004). The present study revealed that hPXR was involved in the complex of chaperones Hsp90 and Hsc70 in the cytoplasm through autophagy (Figs. 3, 6, and 7). Furthermore, the hPXR WT accumulated in CHIP-deleted control cells in the presence of lactacystin (Fig. 7), suggesting that CHIP may play a key role in the proteasome-independent degradation pathway as well as chaperone-dependent quality control (Figs. 3, 6, and 7).

RBCK1 and UBR5 ubiquitinate target proteins for proteasomal degradation in innumerable physiologic reactions; for example, RBCK1 negatively regulates tumor necrosis factor α- and interleukin-1–triggered nuclear factor κB activation by targeting transforming growth factor β–activated kinase 1 binding proteins 2/3 for degradation (Tian et al., 2007), and UBR5 is involved in regulating the stability of the gluconeogenic rate-limiting enzyme phosphoenolpyruvate carboxykinase (Jiang et al., 2011). RBCK1 and UBR5 were previously shown to be responsible for the ubiquitination of hPXR to regulate hPXR degradation through proteasomal machinery (Rana et al., 2013; Ong et al., 2014), whereas the ubiquitin–proteasomal regulation of cytoplasmic CAR retention protein and Hsp70, but not CAR itself, may be an important contributor to the regulation of cytoplasmic CAR levels (Timsit and Negishi, 2014). Unlike RBCK1 and UBR5, CHIP functions in chaperone-dependent ubiquitination for substrate delivery to the proteasome (reviewed in McDonough and Patterson, 2003). Although CHIP ubiquitinated Hsc70 and Hsp70 (Jiang et al., 2001; Soss et al., 2015), but not hPXR, in vitro (Fig. 9), it currently

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**Fig. 10.** Autophagy inhibitor 3-methyladenine (3-MA) led to the greater accumulation of the hPXR T408D mutant than the WT in the presence of proteasome inhibitor lactacystin. HepG2 cells cultured for 24 hours were transfected with expression vectors encoding the Flag-tagged hPXR WT or T408D mutant. Cells were incubated with 5 mM 3-MA or vehicle 12 hours after transfection. Twelve hours later, cells were cultured with lactacystin (5 × 10−4 M) or vehicle for an additional 24 hours. (A) Flag-hPXR, LC3, and p62 proteins in whole cell lysates were determined by Western blotting with the indicated antibodies and anti-α-tubulin antibody (loading control). (B) Band intensities were measured with ImageJ software, and each quantified value of the Flag-hPXR bands was corrected for that of α-tubulin. Relative levels were expressed by taking the values obtained from vehicle-treated cells expressing the Flag-hPXR WT as one. Western blotting was performed three times with similar results, and representative data are shown. Data presented are the average ± S.E. of three experiments. *P < 0.05; **P < 0.01; ***P < 0.001 versus the hPXR WT in vehicle-treated cells; ##P < 0.01; ###P < 0.001 versus the hPXR T408D mutant in vehicle-treated cells; +P < 0.05 versus the hPXR WT in lactacystin- and 3-MA-treated cells; WT, wild-type hPXR; T408D, T408D-mutated hPXR.
Twelve hours later, cells were cultured with lactacystin (5 mM) or vehicle for an additional 24 hours. The subcellular localization of YFP–hPXR was examined by confocal microscopy. Blue, DAPI-labeled nuclei; red, Alexa Fluor 555-labeled LC3 or p62; green, YFP-labeled hPXR. Merged images are shown in the rightmost column and the yellow-stained puncta indicated LC3 or p62, colocalized with hPXR. Experiments were performed twice with similar results, and representative data are shown. Scale bar, 50 μm. WT, wild-type hPXR; T408D, T408D-mutated hPXR.

remains unclear whether CHIP participates in the delivery of the ubiquitinated chaperone complex to the proteasome or autophagic cargo containing p62 or if ubiquitination of the chaperone complex by CHIP induces substrate recognition and recruitment to the proteasome or autophagy. Since Hsc70 ubiquitinated by CHIP is stable (Jiang et al., 2001; Soss et al., 2015), it is conceivable that the ubiquitinated protein may have been detected in the chaperone complex with hPXR (Fig. 9).

Immunocytochemistry revealed that YFP-fused hPXR and p62, which acts as a cargo receptor of ubiquitinated substrates, accumulated and colocalized in cells treated with 3-MA and lactacystin (Figs. 10 and 11). Thus, p62 may play a role in the transport of the ubiquitinated chaperone complex as an acceptor of phagophores in the autophagy-lysosome pathway. Autophagy is a process by which cellular components are delivered to the lysosome for degradation and involves three main pathways: macroautophagy, chaperone-mediated autophagy, and microautophagy (reviewed in Feng et al., 2014). Inhibitions of the proteasome by its inhibitors and Hsp90 by geldanamycin have been shown to induce macroautophagy and chaperone-mediated autophagy, respectively, and ultimately client degradation in the lysosome (Finn et al., 2005; Qing et al., 2006; Ge et al., 2009). We herein demonstrated that inhibition of the lysosome by chloroquine resulted in the accumulation of the hPXR WT and T408D mutant in the cytoplasm (Fig. 8), whereas proteasome inhibition was linked to the enhanced degradation of the T408D mutant because 3-MA suppressed the degradation of hPXR, particularly that of the T408D mutant, in the presence of lactacystin (Figs. 3 and 10). The inhibition of Hsp90 led to the accumulation of the hPXR WT and T408D mutant in the cytoplasm and nucleus (Fig. 6), indicating that the balance of the hPXR quality check by Hsp90 and Hsp70/Hsc70 affected its stability, and also that the T408D mutant was targeted for degradation in a manner that was largely mediated by macroautophagy but not chaperone-mediated autophagy.

PKC cell signaling is known to repress the transcriptional activity of PXR by increasing the strength of the interaction between PXR and the nuclear receptor corepressor protein NCoR (Ding and Staudinger, 2005). We herein demonstrated that PKC directly phosphorylated the threonine-408 of hPXR in vitro (Fig. 12). Although the cellular signaling pathways by which PKC is activated to phosphorylate hPXR in vivo remain unclear, the T408D mutant was degraded faster than the WT in the cytoplasm (Figs. 1, 3, and 6). Thus, PKC may play a key role in regulating the cellular stability of hPXR through phosphorylation at threonine-408. The T408D mutant reduced its stability not only in cultured cancer cells but also in normal cells in mouse livers (Fig. 1E). Therefore, emerging evidence prompted us to propose a schematic model of the regulation of hPXR transactivation and its degradation through the CHIP–Hsc70/Hsp70 protein quality check system (Fig. 13).
Stability Control of PXR via Chaperone-Autophagy Pathway


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Threonine-408 regulates the stability of the human pregnane X receptor through its phosphorylation and the CHIP/chaperone-autophagy pathway

Junko Sugatani, Yuji Noguchi, Yoshiki Hattori, Masahiko Yamaguchi, Yasuhiro Yamazaki, and Akira Ikari

Drug Metabolism and Disposition

Supplemental Fig. 1 Effects of various inhibitors on cell growth in HepG2 cells. HepG2 cells (2 x 10^5 cells) seeded on 6-well plates and cultured for 48 h were treated with proteasome inhibitors (calpain inhibitor I, MG132, and lactacystin), Hsp90 inhibitor (geldanamycin), Hsp70 inhibitor (pifithrin-µ), and lysosomal inhibitor (chloroquine) or vehicle (DMSO) at the indicated concentrations for 24 h. Viable cells were counted using the trypan blue exclusion test. Cell growth in vehicle-treated cells cultured for 72 h (cell number) were taken as one. Data are presented as the average ± S.E. of three experiments. **, p<0.01; ***, p < 0.001 versus the hPXR WT in vehicle-treated cells.
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Drug Metabolism and Disposition

Supplemental Fig. 2  Effects of calpain inhibitor I (A) and geldanamycin (B) on the expression of E3 ligases CHIP, RBCK1, and UBR5 in HepG2 cells. HepG2 cells (2 x 10^5 cells) seeded on 6-well plates and cultured for 24 h were transfected with expression vectors encoding the hPXR WT. The medium was replaced 24 h after transfection, and cells were re-transfected and then cultured with rifampicin (5 x 10^{-6} M) or vehicle (DMSO) in the presence of calpain inhibitor I at 1 x 10^{-5} M, geldanamycin at 1 x 10^{-6} M, or vehicle (DMSO, control) for an additional 24 h. Each mRNA level in cells treated with rifampicin or vehicle in the presence or absence of the inhibitors was expressed by taking the value obtained from hPXR WT-expressing and vehicle-treated control cells as one. Data are presented as the average ± S.E. of three experiments. *, p<0.05; **, p<0.01; ***, p < 0.001 versus the hPXR WT in vehicle-treated control cells; ##. P<0.01; ###, p<0.001 versus the hPXR WT in rifampicin-stimulated control cells.
Threonine-408 regulates the stability of the human pregnane X receptor through its phosphorylation and the CHIP/chaperone-autophagy pathway

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Drug Metabolism and Disposition

Supplemental Fig. 3. p62/SQSTM1 punctate contained the hPXR WT and T408D mutant in HepG2 cells treated with lactacystin and 3-MA. HepG2 cells cultured for 24 h were transfected with expression vectors encoding the YFP-tagged hPXR WT or T408D mutant. Twelve hours after transfection, cells were incubated with 3-MA (5 x 10^-3 M) or vehicle for 12 h, and then were cultured with lactacystin (5 x 10^-6 M) or vehicle for an additional 24 h. The co-localization of the YFP-hPXR and p62 was examined by confocal microscopy. Blue, DAPI-labeled nuclei; Red, Alexa Fluor 555-labeled p62; Green, YFP-labeled hPXR. Merged images are shown in the most right column and the yellow-stained puncta indicated p62, co-localized with hPXR. Experiments were performed twice with similar results, and representative data are shown. Scale bar, 50 µm. WT, wild-type hPXR; T408D, T408D-mutated hPXR.