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Human Constitutive Androstane Receptor Mediates Induction of *CYP2B6* Gene Expression by Phenytoin*

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Compared with its rodent orthologs, little is known about the chemical specificity of human constitutive androstane receptor (hCAR) and its regulation of hepatic enzyme expression. Phenytoin (PHY), a widely used antiepileptic drug, is a potent inducer of CYP2B6 in primary human hepatocytes, but does not activate human pregnane X receptor (PXR) significantly in cell-based transfection assays at the same concentrations associated with potent induction of CYP2B6. Based on this observation, we hypothesized that PHY may be a selective activator of hCAR. In primary human hepatocytes, expression of *CYP2B6* reporter genes containing phenobarbital-responsive enhancer module (PBREM) or PBREM/xenobiotic-responsive enhancer module (XREM) response elements were activated up to 14- and 28-fold, respectively, by 50 μM PHY. By contrast, parallel experiments in HepG2 cell lines co-transfected with an hPXR expression vector did not show increased reporter activity. These results indicated that a PXR-independent pathway, which is retained in primary hepatocytes, is responsible for PHY induction of CYP2B6. Further experiments revealed that PHY effectively translocates hCAR from the cytoplasm into the nucleus in both primary human hepatocytes and CAR$^{-/-}$ mice. Compared with vehicle controls, PHY administration significantly increased *CYP2B6* reporter gene expression, when this reporter construct was delivered together with hCAR expression vector into CAR$^{-/-}$ mice. However, PHY did not increase reporter gene expression in CAR$^{-/-}$ mice in the absence of hCAR vector, implying that CAR is essential for mediating PHY induction of *CYP2B6* gene expression. Taken together, these observations demonstrate that, in contrast to most of the known *CYP2B6* inducers, PHY is a selective activator of CAR in humans.

Induction of cytochrome P450 2B (CYP2B)[1] expression by xenobiotics, including clinically used drugs, is mediated by activation of the nuclear receptors constitutive androstane receptor (CAR) and/or pregnane X receptor (PXR) through response elements located in the promoter region of *CYP2B* genes. In contrast to rodent *CYP2B* genes, mounting evidence suggests that human PXR (hPXR) may be a key receptor in the regulation of human *CYP2B6* gene expression (1–3). hPXR ligands, including rifampicin (RIF), phenobarbital (PB), troglitazone, clotrimazole (CLZ), and SR12813 have been reported to induce *CYP2B6* gene expression (1, 4–7). Recently, fourteen commercially available compounds, including weak, moderate, and strong inducers of CYP2B6 and CYP3A4, were used to compare the potency and magnitude of CYP2B6 and CYP3A4 induction and activation of hPXR in primary cultured hepatocytes and hepatoma cell-based reporter gene assays, respectively. Results from these studies indicate that CYP2B6 induction is highly correlated with the activation of hPXR for most compounds (3). Among the efficacious CYP2B6 inducers evaluated, most activate hPXR but not hCAR, such as RIF and CLZ (CLZ was even reported as a hCAR deactivator by Moore *et al.* (8)), whereas others can activate both hPXR and hCAR, such as PB.

A notable exception is phenytoin (PHY), which exhibits efficacious induction of CYP2B6 protein and mRNA in primary cultured human hepatocytes at 50 μM but relatively weak or nonactivation of hPXR in several human hepatoma cell lines (HepG2 and Huh7) at the same concentration (2). To our knowledge, this would represent the first clinically applied drug that might induce CYP2B6 through a PXR-independent pathway. Our preliminary data demonstrated that PHY could greatly activate *CYP2B6* reporter gene expression in human primary hepatocyte cultures transiently transfected with a *CYP2B6* luciferase reporter construct without co-transfection of exogenous nuclear receptors. Because primary human hepatocytes cultures but not hepatoma-immortalized cells maintain the expression of most nuclear receptors, these results suggest that transcriptional factors other than hPXR may be responsible for PHY induction of CYP2B6 within the range of tested treatment concentrations. Based on these preliminary observations, we hypothesized that hCAR is the predominant regulator of increased *CYP2B6* gene expression by PHY in human hepatocytes.

Although CAR plays an important role in the regulation of

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[1] The abbreviations used are: CYP, cytochrome P450; PB, phenobarbital; RIF, rifampicin; PHY, phenytoin; CLZ, clotrimazole; CITCO, 6-(4-chlorophenyl:imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime; PXR, pregnane X receptor; CAR, constitutive androstane receptor; PBREM, phenobarbital-responsive enhancer module; XREM, xenobiotic-responsive enhancer module; h, human; m, mouse; RT, reverse transcriptase; Bis-Tris, 2-[bis(2-hydroxyethyl)amino]-2-(hydroxymethyl)propane-1,3-diol; YFP, yellow fluorescent protein; EYFP, enhanced YFP; TCPOBOP, 1,4-bis[2-(3,5-dichlorpyridyloxy)]benzene.

xenobiotic-inducible CYP genes, this nuclear receptor exhibits a high level of constitutive transcriptional activity in immortalized cells and can accumulate spontaneously in nuclei (9). Because of the unavailability of a cell system that can effectively sequester CAR in the cytoplasm and decrease its constitutive activation, it is difficult to study the mechanisms of CAR activation. Accordingly, in the current study, we used primary cultured human hepatocytes and CAR knockout mice as alternative systems to evaluate hCAR activation and translocation. Cell line- and primary human hepatocyte-based transfection assays were used to compare the activation profiles of PXR and other nuclear receptors by PHY at the concentrations associated with efficacious induction of CYP2B6 protein and mRNA expression levels in primary human hepatocytes. *In vivo*, PHY activated hCAR in CAR knockout mice co-transfected with a *CYP2B6* reporter gene and an hCAR expression vector. Tail vein injection of a florescence-tagged hCAR plasmid into CAR knockout mice revealed that hCAR is localized in the cytoplasm in untreated mouse livers but translocates to the nucleus after treatment of mice with PHY or other known CAR activators. Taken together, our results demonstrate for the first time that PHY induction of human CYP2B6 is mediated predominantly by CAR rather than PXR.

EXPERIMENTAL PROCEDURES

Materials—Phenytoin, rifampicin, phenobarbital, clotrimazole, dexamethasone, and collagenase type IV were purchased from Sigma. 6-(4-Chlorophenyl:imidazo[2,1-*b*]thiazole-5-carbaldehyde *O*-(3,4-dichlorobenzyl)oxime (CITCO) was obtained from BIOMOL (Plymouth Meeting, PA). Cell culture media and charcoal-stripped fetal calf serum were purchased from Invitrogen. The Dual-Luciferase Reporter Assay System was from Promega Co. (Madison, WI). Oligonucleotides were purchased from Genosys, Inc. (The Woodlands, TX). Real-time RT-PCR probes were from Applied Biosystems (Foster, CA).

Plasmid Constructs—Luciferase-CYP2B6 reporter gene constructs, including pGL3-basic-PBREM, pGL3-basic-PBREM/XREM, and pGL3-tk-(NR1)5 were generated as described previously (2, 10). hPXR expression vectors were obtained from Dr. Binfang Yan (University of Rhode Island). pCR3-hCAR expression vector and fluorescent protein-tagged hCAR (EYFP-hCAR) were constructed as described before (11, 12).

Human Primary Hepatocyte Cultures and Transfection Assays—Liver tissues were obtained by qualified medical staff following donor consent and prior approval from the Institutional Review Board at the University of North Carolina at Chapel Hill. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as previously described (13). Hepatocytes were plated at 3.75×10^5 cells/well into Biocoat® 24-well plates in 0.5 ml of Dulbecco's modified Eagle's medium supplemented with 5% fetal calf serum, antibiotics, insulin, and dexamethasone. After 4 h, cell culture medium was changed to Williams' E medium containing 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium (ITS+) and 0.1 μM dexamethasone.

Transfection experiments were performed 24 h after cell seeding using Effectene® transfection reagent (Qiagen) following the manufacturer's instructions. Briefly, the transfection mixes contained 200 ng of CYP2B6 reporter construct (pGL3 basic-CYP2B6 PBREM or pGL3 basic-CYP2B6 PBREM/XREM) and 25 ng of internal control plasmid (pRL-TK *Renilla* luciferase). Transfected human primary hepatocytes were treated with PHY (10, 20, and 50 μM) or 0.1% Me_2SO control for 24 h, respectively. Subsequently, luciferase activities were measured with hepatocyte lysates using the Dual Luciferase Reporter reagents according to the manufacturer's instructions (Promega).

CYP2B6 induction experiments were performed in hepatocytes cultured in collagen-coated 60-mm Permanox® culture dishes (Nalge Nunc International) as described previously (13). Cultures were incubated for 36–48 h, followed by treatment with PHY (50 μM), RIF (10 μM), PB (1 mM), CLZ (10 μM), and Me_2SO (0.1%) for an additional 72 h. At the end of the treatment period, microsomes and total RNA were isolated as described before (2, 13).

Extraction of Nuclear Proteins—Preparation of nuclear extracts from human primary hepatocytes were carried out as described previously with modifications (14, 15). Briefly, hepatocytes (1×10^7) were scraped into ice-cold phosphate-buffered saline and pelleted by centrifugation at $1500 \times g$ for 5 min. The resulting pellet was resuspended in 1 ml of Buffer A (10 mM HEPES, 10 mM KCl, 1.5 mM $MgCl_2$, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) with 0.3% Nonidet P-40. After keeping on ice for 5 min, the cell suspension was homogenized with a Kontes glass homogenizer (7 ml) B pestle, followed by centrifugation at $1500 \times g$ for 5 min at 4 °C. After repeating the resuspension and centrifugation steps, the supernatant was discarded and 60 μl of lysis buffer (10% glycerol, 10 mM HEPES, pH 7.6, 0.1 M KCl, 3 mM $MgCl_2$, 0.1 mM EDTA, 1 μg/ml pepstatin A, 1 μg/ml leupeptin, 0.5 μg/ml E-46, 1 mM Na_3VO_4, 0.1 mM phenylmethylsulfonyl fluoride, and 1 mM dithiothreitol) was added to the pellet for resuspension. The final concentration was adjusted to 0.4 M by addition of the appropriate volume of 5 M NaCl solution, mixed well, and vigorously shake at 4 °C for 1 h. Finally, the lysates were centrifuged at 14,000 rpm for 10 min at 4 °C, and the supernatant was transferred and stored at −80 °C.

Western Blot and Real-time RT-PCR—Microsomal or nuclear proteins were separated on a NuPAGE Novex 4–12% Bis-Tris gel (Invitrogen) and electrophoretically transferred onto a polyvinylidene difluoride blotting membrane. Subsequently, membranes were probed with specific antibodies against CYP2B6 (Chemicon International) or hCAR (generated as described by Yoshinari *et al.* (14)) and were incubated with horseradish peroxidase-conjugated anti-rabbit IgG. Protein bands were developed with ECL (Amersham Biosciences). For real-time RT-PCR, the total RNA isolated from treated hepatocytes was reverse transcribed to cDNA by using SuperScript II reverse transcriptase (Invitrogen). CYP2B6 primers and *Taq*Man probe were designed using Primer Express software (Applied Biosystems, Foster City, CA) as reported before (2). CYP2B6 forward primer, 5′-AAGCGGATTTGTCTTGGTGAA-3′; reverse primer, 5′-TGGAGGATGGTGGTGAAGAAG-3′; and probe, 6-carboxyfluorescein-CATCGCCCGTGCGGAATTGTTC-6-carboxy-*N*,*N*,*N*′,*N*′-tetramethylrhodamine (TAMRA). For an internal control, β-actin mRNA was measured using Pre-Developed *Taq*Man Assay Reagents for human β-actin (PE Applied Biosystems, Foster City, CA). For quantitative analysis, CYP2B6 mRNA was normalized to β-actin mRNA and expressed as -fold induction and compared with a control mRNA value of one. Amplification and detection were performed using the ABI Prism 7700 Sequence Detector system (Applied Biosystems).

Transfection Assays in Hepatoma-derived Cell Lines—HepG2 and Huh7 cells were cultured in 24-well plates at 5×10^4 cells/well in Dulbecco's modified Eagle's medium, 24 h after seeded cells were co-transfected with hPXR (50 ng), *CYP2B6* reporter vector (100 ng) and pRL-TK (10 ng) as internal control by using Effectene transfection reagent (Qiagen). Transfected cells were treated with PHY (10, 20, and 50 μM), RIF (10 μM), PB (1 mM), CLZ (10 μM), or 0.1% Me_2SO as vehicle control, respectively. 24 h later cells were harvested, and luciferase activity was measured as described above.

In Vivo Gene Transfection and Confocal Imaging Analysis—A CAR-null mouse (16) was first crossbred with C3H to generate CAR heterozygous offspring. Subsequently, CAR heterozygous offspring were repeatedly backcrossed with C3H mice until the genetic background was over 95% C3H. The obtained heterozygous mice were bred to produce the wild-type and CAR-null C3H mice. Animals weighing 23–25 g were quarantined for 1 week before use in temperature- and humidity-controlled rooms with a 12-h light/dark cycle. *CYP2B6* reporter construct (pGL3-basic-PBREM/XREM) (4 μg) with or without pCR3-hCAR vector (5 μg) were injected into $CAR^{-/-}$ mice through the tail vein using TransIT *in vivo* gene delivery system (Mirus, Madison, WI) according to the manufacturer's protocol. Empty pCR3 vector was used to balance the DNA amount in the injection without pCR3-hCAR, and pRL-SV40 (1 μg) was used as internal control. PHY (100 mg/kg), PB (100 mg/kg), CITCO (8 mg/kg), or equal volume of Me_2SO as control was administered intraperitoneally 3 h after gene delivery. Three animals for each group were sacrificed 16 h after the treatment. Livers were homogenized in 5 ml of passive lysate buffer (Promega), and 1 μl of the supernatant obtained by centrifugation at 4 °C was utilized for the dual luciferase assay. For hCAR localization analysis, EYFP-hCAR (10 μg) was tail vein-injected into $CAR^{-/-}$ mice using the same technique described above. Two hours after the injection, PHY (100 mg/kg), PB (100 mg/kg), CITCO (8 mg/kg), or Me_2SO was intraperitoneally administered. A repeat dosage was given by the same route in 3 h. Mice were sacrificed 7 h after tail vein delivery of hCAR. Mouse liver sections were embedded into Tissue-Tek OTC and immediately frozen. Liver frozen slide microscopic detection was carried out as reported previously (12, 17, 18). YFP images from liver sections were analyzed by confocal laser scanning microscopy using a Zeiss LSM510 microscopy system. EYFP-hCAR on the slides was excited at 514 nm, and YFP emission was detected at 530 nm. A 100× oil immersion objective (1.4 numeric aperture) was used for scanning with a pixel size of 0.06 μm. More than 100

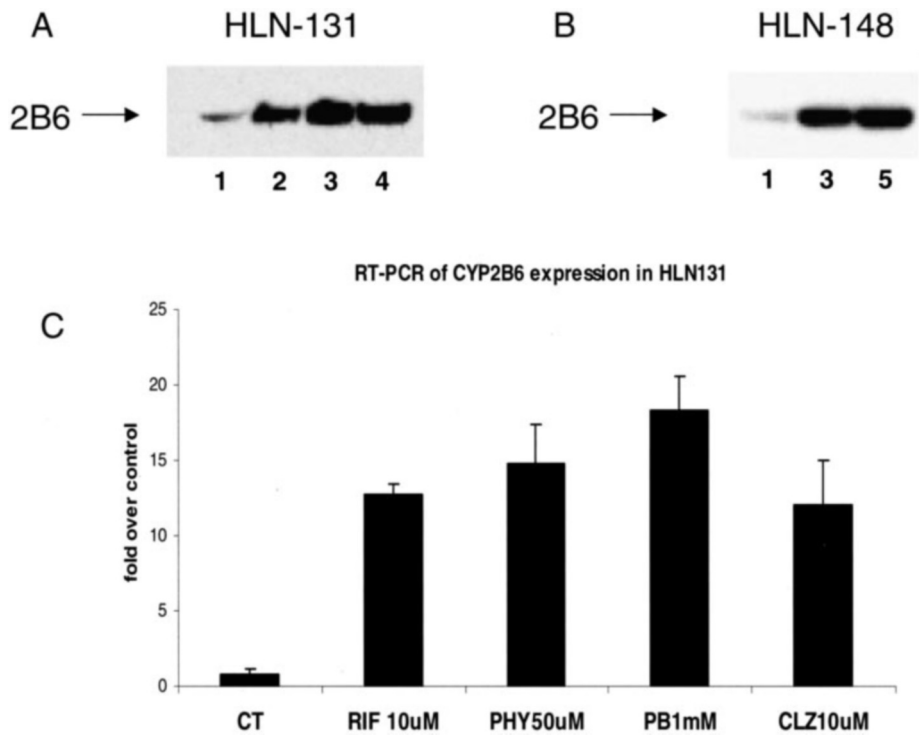


FIG. 1. **Induction of CYP2B6 in primary human hepatocytes.** Human hepatocyte cultures were prepared from two different donors (HLN-131 and HLN-148) and treated for 3 days with RIF (10 μM), PHY (50 μM), PB (1 mM), CLZ (10 μM), or Me_2SO as the vehicle control. Hepatocyte homogenates (25 μg) were prepared for CYP2B6 immunoblot analysis (*A* and *B*). Total RNA extracted from HLN-131 with the same treatments as described above was subjected to real-time RT-PCR analysis of *CYP2B6* mRNA expression level (*C*).

EYFP-hCAR expressed hepatocytes were counted for CAR localization for each group. CAR subcellular localization was characterized as nuclear (N), cytosolic (C), and both nuclear and cytosol (N+C).

RESULTS

PHY Induction of CYP2B6 Gene Expression in Primary Human Hepatocyte Cultures—To compare the extent of CYP2B6 induction by PHY and other prototypical inducers, primary human hepatocytes were treated with PHY, PB, RIF, and CLZ as described under "Experimental Procedures." As shown in two different preparations of human hepatocytes (HLN-131 and HLN-148), 50 μM PHY induced CYP2B6 protein content by 8- and 20-fold, respectively (Fig. 1, *A* and *B*). Real-time RT-PCR analysis showed that CYP2B6 mRNA expression levels were induced 15-, 13-, 18-, and 12-fold over control by PHY, RIF, PB, and CLZ, respectively (Fig. 1*C*). The extent of induction of *CYP2B6* expression in primary hepatocytes after treatment with 50 μM PHY is similar to other prototypical inducers at both the protein and mRNA levels.

Activation of hPXR by Different CYP2B6 Inducers in Hepatoma Cell Lines—Transcriptional activation of the *CYP2B6* gene is mediated synergistically by the PBREM and the distal XREM present in the CYP2B6 promoter (10, 19). To examine a correlation of the CYP2B6 induction in hepatocytes with hPXR activation in hepatoma, *CYP2B6* luciferase reporter constructs containing PBREM, $(NR1)_5$, or PBREM/XREM sequences were co-transfected into Huh7 cells with a hPXR expression vector. In agreement with previous reports (1, 2), the expression of PBREM- or $(NR1)_5$- reporter plasmids was effectively induced by PB, RIF, and CLZ through activation of the co-transfected hPXR. In contrast, little or no activation of hPXR was observed after treatment with 50 μM PHY (Fig. 2, *A* and *B*). In HepG2 cells stably transfected with hPXR, the expression of a transiently transfected CYP2B6 homologous reporter construct containing both the PBREM and XREM was increased after treatment with PB (1 mM), RIF (10 μM), and CITCO (1 and 3 μM) but not after treatment with PHY (20 and 50 μM) (Fig. 2*C*). Based on these observations in Huh7 and HepG2 cells, further investigations were performed in primary hepatocytes and whole animals to determine whether human CAR played a role in mediating PHY induction of CYP2B6.

PHY Activation of CYP2B6 Reporter Gene Expression in Primary Human Hepatocytes—In contrast to cell lines, cultured primary human hepatocytes maintain the expression of most endogenous transcription factors such as nuclear receptors, including CAR, which sequesters in the cytoplasm under basal conditions and translocates into the nucleus in response to direct or indirect activators (12, 20). To examine the effects of endogenous transcription factors on *CYP2B6* reporter gene expression, different *CYP2B6* reporter gene constructs were transfected into two separate preparations of primary human hepatocytes cultured without transfection of exogenous nuclear receptors. As indicated in Fig. 3 (*A* and *B*), 20 μM PHY was associated with 14- and 15-fold increases in the expression of pGL3 basic-PBREM and pGL3 basic-PBREM/XREM reporter constructs, respectively, whereas 21- and 28-fold increases were observed with 50 μM PHY. Notably, the extent of PHY-induced expression of *CYP2B6* reporter activity was similar to the extent of induction of the endogenous *CYP2B6* gene observed in human primary hepatocytes.

PHY Translocates hCAR from Cytosol to Nucleus in Primary Human Hepatocytes—Translocation of CAR from the cytoplasm to the nucleus is the initial step during its activation by xenobiotics (21). The ability to identify efficacious CAR activators using cell-based transfection assays is complicated by the spontaneous nuclear accumulation of CAR in transformed cell lines such as HepG2 and Huh7 (18, 22). To investigate the ability of PHY to activate hCAR, nuclear proteins were extracted from human primary hepatocytes treated with Me_2SO, PHY (50 μM), RIF (10 μM), or PB (1 mM) and subjected to immunoblot analysis. Results showed that PHY and PB treatment accumulated hCAR in the nucleus (Fig. 3*C*). In contrast,

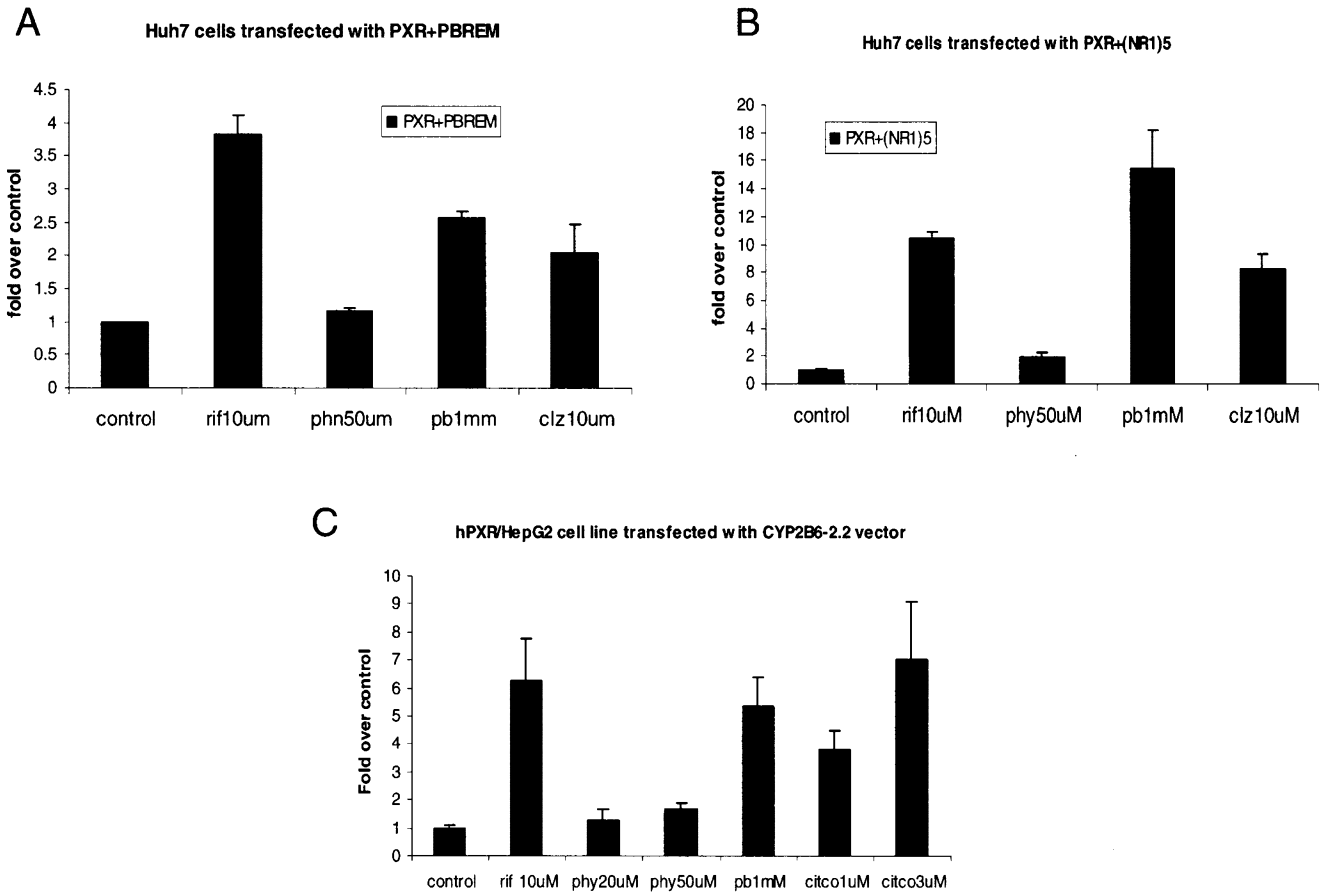


FIG. 2. **Transactivation of *CYP2B6* promoter constructs by human PXR in hepatoma cell lines treated with various inducers.** CYP2B6 reporter vectors containing PBREM or $(NR1)_5$ were transfected into Huh7 cells together with hPXR expression vector as described under "Experimental Procedures." Transfected cells were treated for 24 h with RIF (10 μM), PHY (50 μM), PB (1 mM), CLZ (10 μM), or Me_2SO. Luciferase activities were determined and expressed relative to controls (*A* and *B*). A CYP2B6 reporter construct containing both PBREM and XREM modules was transiently transfected into a HepG2 cell line that stably expresses hPXR. Cells were treated for 24 h with RIF (10 μM), PHY (20 and 50 μM), PB (1 mM), CITCO (1 and 3 μM), or Me_2SO. Luciferase activities were determined and expressed relative to controls (*C*). Data represent the mean ± S.D. ($n = 3$).

RIF (a PXR activator) did not induce translocation of CAR to the nucleus.

PHY Activates and Translocates hCAR in Vivo in Mouse Liver—A previously described mouse tail vein gene delivery technique was used to determine whether *CYP2B6* reporter constructs could be transactivated by hCAR in $CAR^{-/-}$ mice after treatment with PHY (12). Compared with vehicle treatment, PHY, CITCO, and PB resulted in significant increases in the expression of *CYP2B6* reporter gene in $CAR^{-/-}$ mice injected with a *CYP2B6* reporter construct and a hCAR expression vector (Fig. 4). In contrast, there were no significant changes in *CYP2B6* reporter gene expression after the same treatments in $CAR^{-/-}$ mice injected with the *CYP2B6* reporter construct in the absence of hCAR expression vector. These results indicated that PHY activated hCAR in mouse liver, leading to increase *CYP2B6* reporter gene expression. In addition, the absence of effect in $CAR^{-/-}$ mice injected with *CYP2B6* reporter construct alone indicates that other endogenous nuclear receptors play minor roles in regulating PHY induction of *CYP2B6*.

To determine whether PHY-induced expression of *CYP2B6* reporter constructs *in vivo* involved nuclear translocation of hCAR, fluorescent protein-tagged hCAR (EYFP-hCAR) was tail vein-injected into $CAR^{-/-}$ mice. The expression of fluorescently labeled hCAR in the cytosol and nucleus was visualized under a confocal microscopy. Of the hCAR-expressing cells counted in the liver sections of vehicle-treated mice, 11% exhibited hCAR localization in the nucleus, 83% in the cytoplasm, and 6% in the cytoplasm and nucleus (mixed localization). In PHY- and PB-treated mice, the majority of the EYFP-hCAR expression was translocated into the nuclei of hepatocytes (73 and 70%, respectively), whereas cytoplasmic localization decreased to 16 and 19%, respectively. Approximately 11% of the cells showed mixed localization in mice treated with PHY or PB (Fig. 5). In contrast, a previously reported hCAR activator (23), CITCO, showed a relatively lower extent of hCAR nuclear translocation in the current experimental system (33% in the nucleus, 50% in the cytoplasm, and 17% as mixed localization) (Fig. 5). Overall, there was an approximate 70% increase in the nuclear localization of hCAR after PHY or PB treatment in $CAR^{-/-}$ mice. These results indicate that PHY is capable of inducing both the nuclear translocation and activation of hCAR in the mouse livers.

DISCUSSION

CAR regulation of *CYP2B* genes expression has been well established in rodents (24, 25). Mouse CAR (mCAR) activators such as TCPOBOP and PB can induce *Cyp2b10* gene expression in both intact mouse liver and primary cultured mouse hepatocytes. Induction of *Cyp2b10* expression can be blocked effectively by the mCAR repressors androstanol and androstenol. However, current knowledge is limited regarding the role of hCAR in the regulation of human *CYP2B* gene expression. To date, accumulated evidence suggests that the human

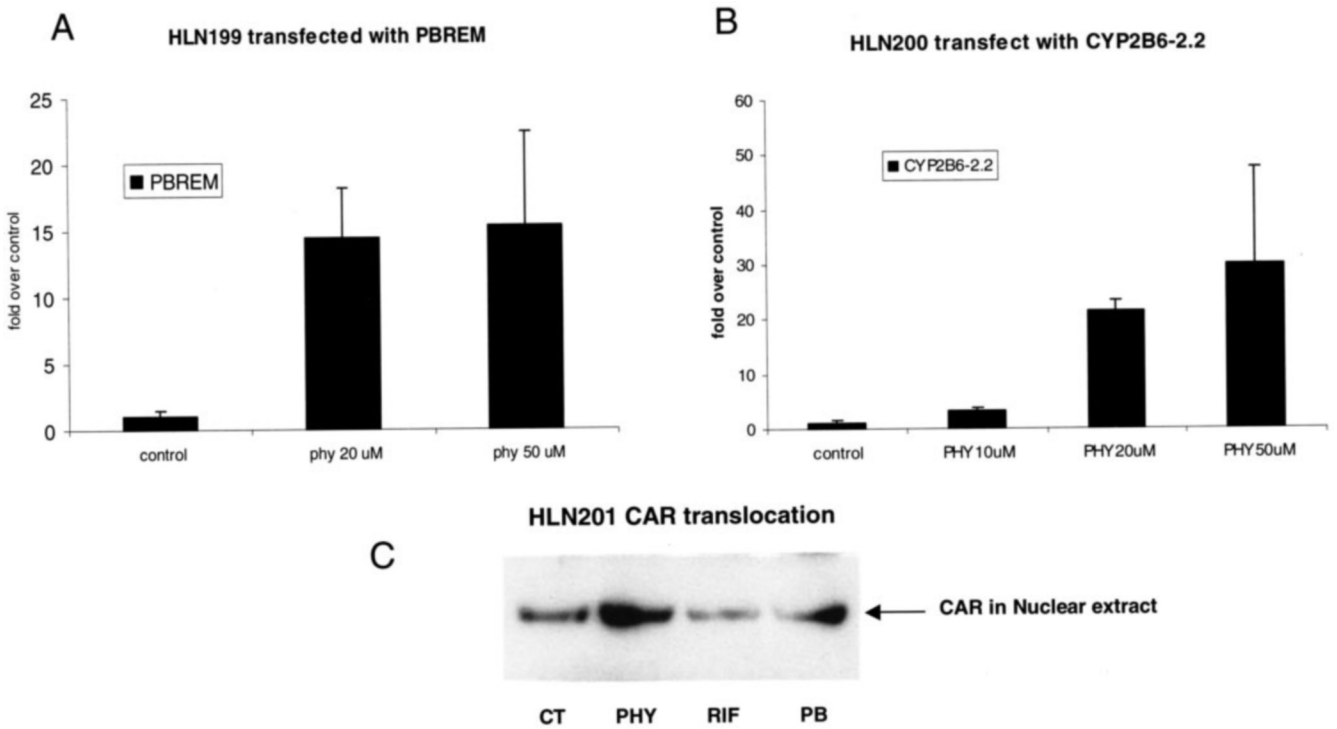


FIG. 3. **PHY increases *CYP2B6* reporter gene expression and translocates hCAR in primary human hepatocytes culture.** Primary human hepatocytes from two liver donors (HLN-199 and HLN-200) were seeded onto 24-well plates. Hepatocytes were transfected with *CYP2B6* reporter vectors (pGL3-basic-PBREM or pGL3-basic-PBREM/XREM) then treated with PHY (10, 20, or 50 μM). Dual luciferase activities were measured following the manufacturer's protocol (*A* and *B*). Hepatocytes cultured in 60-mm dishes were treated for 3 days with PHY (50 μM), RIF (10 μM), PB (1 mM), or Me_2SO. Nuclear extracts, prepared as described under "Experimental Procedures," were subjected to Western immunoblot analysis using hCAR antibody (*C*).

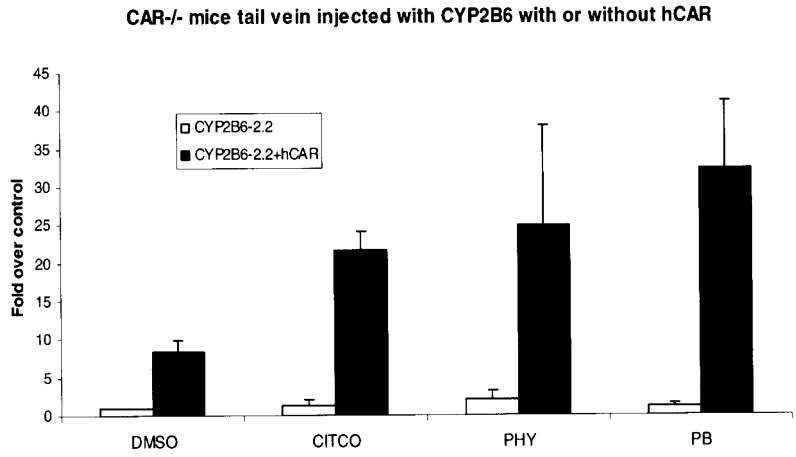
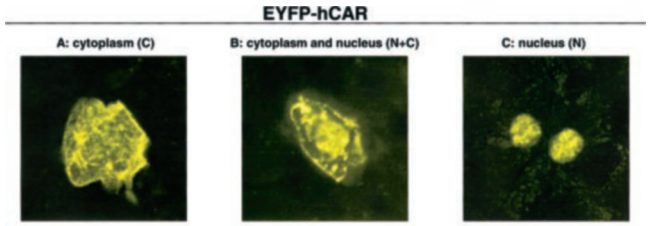


FIG. 4. **Drug-induced expression of a *CYP2B6* reporter construct in $CAR^{-/-}$ mice in the presence and absence of hCAR.** A *CYP2B6* promoter construct (pGL3-basic-PBREM/XREM) was delivered into mice by tail vein injection, in the presence or absence of hCAR expression vector, by using the TransIT *In Vivo* Gene Delivery System as described under "Experimental Procedures." Three hours after the injection, PHY (100 mg/kg body weight), PB (100 mg/kg body weight), CITCO (8 mg/kg body weight), or Me_2SO was administered intraperitoneally. Animals were sacrificed 16 h afterward, and dual luciferase assays were performed on liver lysates. Data represent the mean ± S.D. for each treatment group (n = 3 animals per group).

CYP2B6 gene is regulated in a PXR-dependent manner (1, 3, 26). The majority of known PXR activators are capable of potently inducing CYP2B6 in primary human hepatocyte cultures, including those that activate hPXR only (rifampicin, hyperforin, and SR12813), those that activate both hPXR and hCAR (PB), or those that activate hPXR but deactivate hCAR (CLZ and ethynyl estradiol) (1, 3, 27). To our knowledge, the current study is the first to demonstrate that a clinically used drug such as PHY can induce CYP2B6 through a PXR-independent pathway.

In vitro cell-based reporter assays have been used extensively for screening chemical entities as activators of various nuclear receptors (8, 28). Without exception in previous studies, compounds that activated hPXR in reporter assays in hepatoma cell lines also induced CYP2B6 in primary human hepatocytes. Conversely, compounds that failed to activate did not induce CYP2B6 (3). However, this study identifies PHY as an exception to these previous observations. At 50 μM, PHY induces CYP2B6 in primary human hepatocytes with similar efficacy as several prototypical inducers, including PB, RIF, and CLZ. However, this same concentration of PHY does not activate hPXR significantly in either HepG2 or Huh7 cells as assessed by *CYP2B6* reporter gene activity. These results are in agreement with a previous report by Raucy *et al.* (29), where only minor activation of PXR was observed with PHY at concentrations up to 1 mM in HepG2 cells (29). These initial results provided the first inclination that hPXR may not be the primary mediator of PHY induction of *CYP2B6* gene expression.

Compared with transformed cells, human primary hepatocyte cultures have been recognized as a more reliable *in vitro* model for evaluating the induction of drug-metabolizing enzymes in human liver (30, 31). One possible reason for the maintenance of xenobiotic inducibility of drug-metabolizing enzymes in primary but not transformed cells is the retention of



	N	N+C	C	Total
Control	13 (11%)	8 (6%)	101(83%)	122
CITCO	40 (33%)	20 (17%)	60 (50%)	120
PHY	110 (73%)	17 (11%)	24 (16%)	155
PB	75 (70%)	12 (11%)	20 (19%)	107

FIG. 5. **Cellular localization of fluorescent protein-tagged hCAR in CAR$^{-/-}$ mice.** A fluorescent-hCAR (EYFP-hCAR) was tail-vein injected into CAR$^{-/-}$ mice, treated with PHY (100 mg/kg body weight), PB (100 mg/kg body weight), CITCO (8 mg/kg body weight), or Me_2SO intraperitoneally. Animals were sacrificed 7 h later, and frozen liver sections were prepared for analysis by confocal microscopy as described under "Experimental Procedures." Images of liver sections from mice treated with solvent (*A*), CITCO (*B*), and PHY (*C*) illustrating EYFP-hCAR localization as: cytoplasm (*C*); mixed cytoplasm and nuclear (*N+C*); or nuclear (*N*). The table shows the percentage of cells exhibiting the different types of hCAR localization in mouse liver sections taken from each treatment group.

endogenous transcription factors in the former but not latter *in vitro* model. In contrast to the results obtained from hepatoma cell lines, treatment of primary hepatocytes with 20 or 50 μM PHY resulted in robust increases in CYP2B6 reporter expression in the absence of exogenously transfected nuclear receptor. In agreement with our previous report, greater increases were observed with transfection of the PBREM/XREM reporter construct compared with the construct containing the PBREM alone (10). These results in primary hepatocytes indicate that PHY induction of CYP2B6 is regulated by transcription factors other than PXR. Furthermore, these observations suggest that primary hepatocytes are superior to cell lines in elucidating the mechanisms regulating xenobiotic-mediated CYP gene expression due to retention of endogenous cellular and nuclear cofactors required for transcriptional control.

Because studies in human hepatocytes implicated the role of a nuclear receptor other than PXR in PHY-mediated induction of CYP2B6, we considered hCAR as another likely candidate because of the ability of its rodent counterpart to regulate CYP2B induction and the known cross-regulation between it and hPXR. Unlike the case for rodent CAR, greater difficulties have been encountered in the evaluation of human CAR regulation of target genes. Similar to its rodent counterparts, hCAR spontaneously translocates to the nucleus in hepatoma cell lines such as HepG2 and Huh7 independently of ligand binding and/or activation. However, in contrast to its rodent counterparts, hCAR is not inhibited by mCAR repressors, such as androgens, androstanol, progesterone, and calmodulin kinase inhibitors (21). Furthermore, specific ligands identified for mCAR, such as PB and TCPOBOP, have less selectivity for the human receptor.

Although there are difficulties in using immortalized cells for evaluating CAR function and activity, as described above, it appears that primary human hepatocytes represent an alternative *in vitro* system for this purpose. Studies have shown that CAR is compartmentalized in the cytoplasm of primary hepatocytes and *in vivo* in the absence of ligands and/or activators (12). Translocation from the cytoplasm to the nucleus has been considered the initial step of its activation. In experiments conducted with human primary hepatocytes, we demonstrated that PHY induces translocation of hCAR from the cytoplasm to the nucleus to a similar extent as the known CAR activator PB, whereas RIF, a known PXR activator, does not. These observations indicate that PHY can initiate the first required step of CAR activation in primary hepatocytes, leading to eventual up-regulation of *CYP2B6* gene expression. To further evaluate the role of hCAR in the regulation of PHY-induced *CYP2B6* gene expression, CAR$^{-/-}$ mice were transiently transfected with CYP2B6 reporter vectors alone or in combination with hCAR expression vector. Results from these *in vivo* experiments demonstrated that CAR is required for PHY activation of CYP2B6 reporter gene expression. In the absence of hCAR, PHY and other known CAR activators failed to increase the expression of the transfected *CYP2B6* reporter gene, indicating that other endogenous transcription factors in mice, including PXR, are not capable of mediating PHY-induced *CYP2B6* reporter gene expression *in vivo*. Using CAR-null mice, a recent study by Jackson *et al.*[2] identified PHY as a new mCAR activator involved in the induction of murine *Cyp2b10* and *Cyp2c29*. PHY induces both *Cyp2b10* and *Cyp2c29* expression in wild-type mice, but the induction was dramatically reduced in CAR$^{-/-}$ mice. These results suggest that PHY induces murine *Cyp2b10* and *Cyp2c29* primarily via the nuclear receptor CAR.

Confocal microscopic analysis using fluorescence-tagged hCAR further confirmed that the majority of transfected hCAR is expressed in the cytoplasm in the absence of CAR activators, but that PHY and PB can induce efficient translocation of hCAR into the nucleus. Therefore, PHY appears to be a selective activator for both human and mouse CAR. On the contrary, CITCO, previously reported as a potent human CAR activator (23), demonstrated a moderate capacity for fluorescence-tagged hCAR nuclear translocation compared with PHY and PB. About 11% of the nuclei expressed fluorescence-tagged hCAR in the control animals, which may have contributed to the higher basal expression of *CYP2B6* reporter gene activity compared with mice that received the *CYP2B6* reporter gene alone. In contrast to PXR, which requires direct ligand binding for activation, CAR can be activated directly by compounds such as TCPOBOP and CITCO, or indirectly by compounds such as PB (8). An *in vitro* ligand binding assay indicated that PHY does not bind hCAR or with very weak affinity,[3] suggesting that PHY activates hCAR through an indirect pathway in a manner similar to PB.

PHY is a widely prescribed anticonvulsant drug used for the prevention and treatment of seizures. Because it often is prescribed chronically and co-administered with other therapeutics, drug-drug interactions associated with PHY are common (32, 33). In humans, PHY is predominantly metabolized by the cytochrome P450 2C isozymes (~80% by CYP2C9 and 20% by CYP2C19) and is a recognized inducer of CYP2B6, CYP2C9, and CYP3A4 (34–36). Because more than 40% of patients with brain tumors experience disease-related seizures (37, 38), drug-drug interactions between anticancer agents and antiepileptic drugs such as PHY can have significant clinical consequences (39). It has been reported that PHY induction of CYP2B6 results in altered pharmacokinetics of the antineoplastics cyclophosphamide and ifosfamide, which are frequently co-administered with PHY (40, 41). Thus, PHY induction of liver cytochrome P450 expression can result in decreased efficacy of co-administered chemotherapeutics metabolized by CYP2B6. However, to date, the mechanisms underlying PHY induction of CYP2B6 have not been elucidated. Based on the results of these studies, PHY is the first drug identified to cause drug

[2] Jackson, J. P., Ferguson, S. S., Moore, R., Negishi, M., and Goldstein, J. A. (2004) *Mol. Pharmacol.* **65,** 1397–1404.

[3] B. Goodwin, personal communication.

interactions predominantly through activation of the nuclear receptor CAR.

In summary, our data suggest that PHY induction of *CYP2B6* gene expression in human primary hepatocytes is mediated predominantly through activation of human CAR, not hPXR, indicating that multiple classes of CYP2B6 inducers exist. PHY is the first clinically used drug to be identified as inducing CYP2B6 through a PXR-independent pathway. Moreover, reporter assays conducted in primary human hepatocytes most reliably reflect P450 regulation *in vivo* both qualitatively and quantitatively compared with those conducted in cell lines.

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