GLUCOCORTICOID RECEPTOR ENHANCEMENT OF PREGNANE X RECEPTOR-MEDIATED CYP2B6 REGULATION IN PRIMARY HUMAN HEPATOCYTES

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ABSTRACT:

Although the glucocorticoid receptor (GR) facilitates the xenobiotic-induced expression of CYP2B in rodents, its role in the regulation of human CYP2B6 is unclear. In this report, the role of human GR in the regulation of CYP2B6 was evaluated using primary human hepatocytes and transfection assays with HuH7 cells. CYP2B6 expression was not induced in primary hepatocytes treated with dexamethasone (DEX) concentrations (0.01–1 μM) known to activate GR. In contrast, treatment with 0.1 μM DEX enhanced CYP2B6 induction by different pregnane X receptor (PXR) activators, including rifampin, phenytoin, clotrimazole, and phenobarbital. In HuH7 cells, cotransfection of human (h)GR and hPXR with CYP2B6-phenobarbital-responsive enhancer module (PBREM) reporter constructs revealed that all hPXR ligands induce CYP2B6 reporter gene activity, and this ligand-dependent activation is greatly enhanced by activated hGR. CYP2B6 reporter gene expression was not induced in the presence of hPXR ligands when hGR alone was cotransfected with CYP2B6 reporter construct. In hGR and human constitutive androstan receptor (hCAR) cotransfection assays, activated hGR increased the constitutive activation of PBREM reporter constructs by hCAR in the absence of inducers. In the presence of activated hGR and known inducers of CYP2B6, only PB treatment caused a further 2-fold activation of hCAR compared with control. These studies show that hGR is involved synergistically in the xenobiotic-responsive regulation of human CYP2B6 by hPXR and hCAR. Moreover, the results suggest that the GR-enhanced expression of CYP2B6 is mediated through an indirect mechanism that does not require increased expression of nuclear receptor.

Cytochrome P450s (P450s) constitute a superfamily of heme-containing monooxygenases that catalyze the biotransformation of a broad range of endogenous and xenobiotic chemicals (Nelson, 1999; Parkinson, 2001). CYP2B6 has been thought to play a minor role in human drug metabolism and xenobiotic biotransformation; therefore, it has received little attention in pharmacology and toxicology (Mimura et al., 1993; Shimada et al., 1994). This concept has been challenged by recent studies that estimate the average relative abundance of CYP2B6 to be at least 5% of the total hepatic P450 content and by the fact that up to 25% of all pharmaceutical drugs are metabolized to some extent by CYP2B6 (Code et al., 1997; Ekins, 1999; Stresses, 1999; Faucette et al., 2000; Hanna et al., 2000; Hesse et al., 2000). Also of importance, CYP2B6 is highly inducible and significant interindividual differences in hepatic CYP2B6 expression exist (Xie and Evans, 2001). However, the molecular determinants of human CYP2B6 expression and regulation by xenobiotics remain unclear.

Transcriptional activation of CYP genes by xenobiotics is mediated by the interaction of ligand-nuclear receptor complexes with enhancer sequences that lie upstream of CYP gene promoters (Kliewer et al., 1999; Waxman, 1999). Recently, pregnane X receptor (PXR; NR1I2) and constitutive androstan receptor (CAR; NR1I3) have been proposed as xenobiotic-responsive transcription factors that regulate multiple drug-metabolizing enzymes (Bertilsson et al., 1998; Xie et al., 2000; Geick et al., 2001). Extensive studies by Negishi and coworkers have revealed that CAR regulates the induction of rodent CYP2B gene expression by phenobarbital (PB) and PB-like compounds (Honakoski et al., 1998; Kawamoto et al., 1999). In studies performed in CAR knockout mice, total ablation of Cyp2b10 induction by PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is observed (Wei et al., 2000; Ueda et al., 2002). Although human CAR shares many common characteristics with its rodent counterparts, it also displays unique ligand specificities, as well as other distinct properties (Kawamoto et al., 2000).

PXR is the closest relative of CAR within the orphan nuclear receptor superfamily. Although they are originally recognized as the regulator of CYP3A and CYP2B, respectively, significant overlap is observed between CYP2B and CYP3A induction by the same inducers (Xie et al., 2000; Goodwin et al., 2001). The mechanism under-
lying the shared induction of CYP2B and CYP3A currently is unknown, but may result from the cross talk of CAR and PXR by recognition of common response elements (Xie et al., 2000).

In contrast to hCAR and hPXR, the role of GR in the CYP2B6 basal and xenobioto-induced expression in human liver has not been extensively explored. GR is activated upon binding of glucocorticoids in various tissues and can regulate gene transcription by activation as well as repression (Beato et al., 1995). Submicromolar concentrations of dexamethasone (DEX) known to activate hGR do not induce CYP2B6 expression at the mRNA level (Pascussi et al., 2000b). Using a GR knockout animal model, Schuetz et al. (2000) demonstrated that GR-deficient mice challenged with DEX failed to induce CYP2B proteins, whereas CYP2B6 was readily induced in wild-type mice by DEX (Schuetz et al., 2000). Furthermore, the basal hepatic expression of CYP2B6 was decreased in mice lacking GR, whereas the constitutive expression of liver CYP3A was not affected (Schuetz et al., 2000). Given these findings, it is clear that GR plays an important role in both the basal and inducible expression of CYP2B6 by glucocorticoids in mice. However, the role of GR in the overall regulation of human CYP2B6 gene expression remains to be determined.

In the current study, we report the roles of GR and hPXR in the regulation of CYP2B6 expression in primary cultures of human hepatocytes. Different hPXR activators such as rifampicin (RIF), PHY, phenytoin (PHY), and clotrimazole (CLZ) were chosen to evaluate the relationship of hPXR activation and CYP2B6 induction. In addition, the presence or absence of DEX cotreatment was examined for its effect on PXR-mediated regulation of CYP2B6. Furthermore, CYP2B6 and CYP3A4 mRNA and protein expression patterns, and PXR mRNA expression levels were compared in human primary hepatocytes treated with DEX (0.001–1 μM). Cotransfection of GR and hPXR or hCAR with pHBREM or NR1 reporter vectors in Huh7 cells was performed to examine whether hPXR ligands activate CYP2B6 PPREM reporter gene expression, and whether it is enhanced by the activation of exogenous GR. The results from these studies suggest that hPXR may play an important role in the regulation of CYP2B6, and this effect is greatly facilitated by GR activation. Moreover, GR alone does not directly regulate CYP2B6 expression in human primary hepatocytes, but rather seems to work synergistically with hPXR and hCAR to regulate xenobioto-responsive CYP2B6 expression.

Materials and Methods

Materials and Reagents. DMEM and modified Che’s medium (MCM) were purchased from Invitrogen (Carlsbad, CA). Matrigel and ITS+ were obtained from Collaborative Research (Bedford, MA). Collagenase IV was from Sigma-Aldrich (St. Louis, MO) and petri dishes (60 mm, LUX, Permanox) were from Nalge Nunc International (Naperville, IL). DEX, PB, RIF, PHY, CLZ, bupropion hydrochloride (BUP), NADP+, glucose 6-phosphate, and glucose-6-phosphate dehydrogenase were purchased from Sigma-Aldrich. Effectene transfection reagent was obtained from QIAGEN (Valencia, CA). Dual-luciferase reporter assay system was from Promega (Madison, WI). All other reagents were purchased from commercial suppliers and were either American Chemical Society or molecular biology grade.

Plasmids. The expression vector for human PXR was generously provided by Dr. Bingfang Yan (University of Rhode Island, Kingston, RI). Human GR expression vector was a kind gift from Dr. John Cidlowski (National Institute of Environmental Health Sciences, Research Triangle Park, NC). Human CAR expression vector and the reporter construct with the human PBREM cloned in front of the tk promoter termed hPBREM-tk-LUC were as described by Sueyoshi et al. (1999). A 5-repeat human NR1 (5¢-gatcACTGTACTTTCCT-GACCTTGgatc-3¢) sequence was synthesized and cloned into the KpnI/XhoI site of pGL3-promoter vector, resulting in a (NR1)5-LUC plasmid.

Culture and Treatment of Primary Human Hepatocytes. Hepatocytes were isolated from human liver specimens by a modification of the two-step collagenase digestion method as described by Hamilton et al. (2001). Liver tissues were obtained by qualified medical staff, with donor consent and the approval of the University of North Carolina Hospitals Ethics Committee. All tissues were obtained from donors undergoing liver resections for metastatic tumors. Only hepatocytes exhibiting normal morphological features and viabilities greater than 80% were plated into collagen-coated 60-mm Permanox culture dishes (Nalge Nunc International) at the density of 4 × 105 cells/dish in 3 ml of serum-free MCM containing 6.25 μg/ml insulin, 6.25 μg/ml transferrin, and 6.25 ng/ml selenium (ITS+). After 2 to 4 h of attachment at 37°C in a humidified incubator with an air:CO2 ratio of 95:5, cultures were overlaid with ice-cold medium containing 0.25 mg/ml Matrigel. Medium containing the appropriate concentration of DEX as outlined under Results was changed on a daily basis thereafter. Cultures were incubated for an additional 36 to 48 h before initiating experiments with inducers.

Hepatocyte cultures (n = 3–5 dishes/treatment group) were treated for three consecutive days with test compounds at final concentrations of 1 and 10 μM for RIF, 100 and 1000 μM for PB, 50 μM for PHY, and 10 μM for CLZ in the presence or absence of 0.1 μM DEX. Control cultures were treated with 0.1% DMSO. In separate studies, cultures of human hepatocytes were treated with medium containing different concentrations of DEX from 0.001 to 1 μM for the same period of time as described above. At the end of the treatment period, cells were harvested and microsomes were prepared as described previously (Hamilton et al., 2001). Protein concentration was determined with the biocinchoninic acid protein assay kit (Pierce Chemical, Rockford, IL) according to the manufacturer’s instructions.

Measurement of Bupropion and Testosterone Hydroxylase Activities. The activity of CYP2B6 was determined by measuring the rate of hydroxylation of BUP (Faucette et al., 2000). Briefly, microsomes isolated from cultured human hepatocytes (0.1 mg) were incubated at 37°C in 0.25-ml incubation mixtures containing potassium phosphate buffer (50 mM, pH 7.4), MgCl2 (3 mM), EDTA (1 mM), NADP+ (1 mM), glucose 6-phosphate (5 mM), glucose-6-phosphate dehydrogenase (1 unit/ml), and BUP (500 μM). Reactions were started by the addition of the NADPH-generating system and terminated 30 min later by addition of ice-cold acetonitrile (125 μl). On completion of the reaction, reaction mixtures were vortexed and centrifuged at 2000g for 5 min. The amount of hydroxy-BUP in the supernatant fraction was determined by C18 reverse-phase high-performance liquid chromatography preceded by a BDS-Hypersil C18 guard cartridge using UV detection set at 210 nm. Elution was achieved at 1 ml/min using a binary gradient of solvent A (0.025M potassium phosphate, 1 mM triethylamine, pH 7.0 in 95:5 water/acetonitrile) and solvent B (acetonitrile). CYP3A4 activity was determined by measuring the production of 6-hydroxytestosterone as described by Pearce et al. (1996). SDS-Polyacrylamide Gel Electrophoresis and Immunoblotting Analysis. Microsomal protein (10–30 μg) was separated by SDS-polyacrylamide gel electrophoresis (9%), and electrotherophoretically transferred to polyvinylidene difluoride blot membrane. Membranes were probed with specific antibodies against CYP2B6 or CYP3A4 peptide (Chemicon International), and developed with an enhanced chemiluminescence detection system (Amersham Biosciences Inc., Piscataway, NJ). The relative amounts of CYP2B6 and CYP3A4 protein were estimated from densitometry analysis of the blot after scanning.

Transfection of Hepatoblastoma Cells. A human liver-derived cell line, HuH7, was cultured in DMEM/F-12 supplemented with 10% charcoal stripped fetal bovine serum (HyClone Laboratories, Logan, UT) and antibiotics. Briefly, 5 × 105 cells/well were plated into 24-well plates and transfected 24 h later with Effectene transfection reagent (QIAGEN). Transfection mixtures consisted of 100 ng of hPBREM-LUC or (NR1)5-LUC reporter constructs, 50 ng of hPXR or hCAR with and without 50 ng of hGR cotransfection, and 10 ng of pRL-tk vector as internal control. Cells were maintained for a further 24 h in the presence of different combinations of compounds in DMEM/F-12. Luciferase activity was measured using the dual-Luciferase reporter assay system (Promega). Promoter activities were determined from three independent transfections and calculated from firefly luciferase activities normalized against Renilla luciferase activities of the internal control pRL-tk vector.

Real-Time PCR. Total RNA was isolated from human hepatocytes using RNease Midi kit (QIAGEN). cDNAs were prepared from total RNA using SuperScript II reverse transcriptase (Invitrogen). The primers and the TaqMan probes were designed using Primer Express software (Applied Biosystems, Foster City, CA) from the human mRNA sequence as follows: CYP2B6 1299

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Human hepatocytes (HLN-126) cultured in MCM were treated with different hPXR ligands, including: RIF (10 μM), PHY (50 μM), CLZ (10 μM), PB (1 mM), or vehicle (0.1% DMSO), respectively, for 72 h before harvest. A, microsomal proteins (30 μg) were analyzed for bupropion hydroxylase activity. Similar induction patterns as in A were observed in CYP2B6 activities by hPXR activators.

Results

Various PXR Ligands Induce CYP2B6 Expression and Activity in Primary Human Hepatocyte Cultures. Microsomes isolated from a single preparation of human hepatocytes (HLN-126) treated with a panel of known PXR ligands for 72 h were analyzed for CYP2B6 protein content and activity by immunoblotting and BUP hydroxylase activities were significantly correlated with CYP2B6 hydroxylase activities were significantly correlated with CYP2B6. In this study, the rate of BUP hydroxylation was used as a marker of CYP2B6 catalytic activity in microsomes isolated from human hepatocytes treated with different compounds (Faucette et al., 2000).

Effects of Submicromolar Levels of DEX on CYP2B6, CYP3A4, and PXR Expression. Initial studies using human primary hepatocyte cultures (HLN-109) demonstrated that different concentrations of DEX from 0.001 to 1 μM did not induce CYP2B6 protein and mRNA expression (Figs. 1A and 1B). It has been shown that this range of DEX (hereafter referred to as low DEX concentrations) activates GR but not PXR or CAR (Pascussi et al., 2000a; Falkner et al., 2001). In parallel experiments, CYP3A4 expression was also analyzed under the same low concentrations of DEX. In contrast with CYP2B6, both CYP3A4 mRNA and protein expression were induced in a dose-dependent manner by DEX concentrations between 0.01 and 1 μM (Figs. 2B and 3B). These notably different profiles for CYP2B6 and CAR and PXR. However, potent induction of CYP2B6 was also observed with two potent PXR activators RIF and CLZ. Because CLZ is a PXR activator but a CAR deactivator (Moore et al., 2000), induction of CYP2B6 by RIF and CLZ suggests that PXR also may play an important role in the regulation of CYP2B6.

Enhancement of CYP2B6 Induction by PXR Ligands in Presence of DEX. To determine whether DEX cotreatment enhances CYP2B6 induction by PXR ligands, hepatocyte cultures were maintained in medium with or without 0.1 μM DEX before and during treatment with different PXR ligands. The results showed that DEX enhanced the CYP2B6 protein content and activity of hepatocytes treated with all compounds tested in this study (Fig. 4, A and B). On the other hand, real-time PCR results indicated that under the treat-
Recent studies have indicated that in vitro translated CAR (Sueyoshi et al., 1999). The CYP2B6 gene contains two imperfect DR4 elements (NR1 and NR2), which were identified as the most important response elements of the CYP2B6 gene. PBREM located 1.7 kilobases upstream of the 5′-flanking region of the CYP2B6 gene contains two imperfect DR4 elements (NR1 and NR2), which were identified as the most important response elements mediating transcriptional activation of CYP2B6 by CAR (Sueyoshi et al., 1999). Recent studies have indicated that in vitro translated PXR-RXR heterodimers also could bind to the NR1 and NR2 within the PBREM region (Xie et al., 2000; Goodwin et al., 2001). To test the hypothesis that activated GR could enhance PXR regulation of CYP2B6 gene expression by facilitating PXR activation of CYP2B6 reporter constructs, experiments were carried out in a liver-derived cell line (Huh7) transiently transfected with various plasmids.

**Cotransfection of GR with PXR Synergistically Activates CYP2B6 Reporter Constructs by DEX and PXR Ligands.** A 51-bp PBREM located 1.7 kilobases upstream of the 5′-flanking region of the CYP2B6 gene contains two imperfect DR4 elements (NR1 and NR2), which were identified as the most important response elements mediating transcriptional activation of CYP2B6 by CAR (Sueyoshi et al., 1999). Recent studies have indicated that in vitro translated PXR-RXR heterodimers also could bind to the NR1 and NR2 within the PBREM region (Xie et al., 2000; Goodwin et al., 2001). To test the hypothesis that activated GR could enhance PXR regulation of CYP2B6 gene expression by facilitating PXR activation of CYP2B6 reporter constructs, experiments were carried out in a liver-derived cell line (Huh7) transiently transfected with various plasmids.

Without exogenous GR, cotransfection of PXR expression vector with the hPBREM-LUC reporter plasmid resulted in modest increases in the luciferase reporter gene expression by various PXR ligands (Fig. 5A). PB (1 mM) and RIF (10 μM) resulted in 2-fold activation, with the normalized luciferase ratio (firefly/Renilla) of ~25 units. Under these transfection conditions, cotreatment with 0.1 μM DEX did not result in any further activation by these compounds (Fig. 5A). In contrast, upon cotransfection of GR with hPXR and hPBREM-LUC, 0.1 μM DEX greatly enhanced PXR-ligand-mediated hPBREM-LUC reporter gene expression. The reporter activity with RIF (10 μM) and 0.1 μM DEX was approximately 5-fold higher than that with RIF (10 μM) alone, with a normalized luciferase ratio of 110 units (Fig. 5B). However, in the absence of 0.1 μM DEX, there was no difference in the activation patterns and potency of various PXR ligands in the presence and absence of GR cotransfection. These results indicate that DEX facilitates PXR-mediated activation of CYP2B6 expression by a GR-dependent process rather than by a nonspecific mechanism.

The relative contributions of NR1 and NR2 motifs within the PBREM to the transcriptional activation of CYP2B6 has been compared in several studies, and it has been suggested that quantitatively the NR1 motif plays a more important role in CYP2B regulation (Honkakoski et al., 1998; Goodwin et al., 2001). In this study, a pGL3-(NR1)5-LUC reporter construct was transiently transfected into Huh7 cells with the same PXR and GR constructs as those used in the hPBREM-LUC experiments. Figure 6A shows that without GR cotransfection, there is no additional activation observed in the presence of 0.1 μM DEX cotreatment with PXR ligands compared with that without DEX cotreatment. In the presence of exogenous GR, 0.1 μM DEX markedly increased PXR activation of the reporter gene by PXR ligands. The highest increase was observed with PB (1 mM) and RIF (10 μM) in the presence of 0.1 μM DEX, which were 5- and 4-fold higher, respectively, compared with that in the absence of DEX cotreatment (Fig. 6B). These results were similar to those observed in the hPBREM-LUC experiments and indicate that NR1 alone accounts for most of the PXR-mediated activation of the PBREM vector, and suggest that it is the most important element in the GR and PXR coregulation of the PBREM. Notably, cotransfection of Huh7 cells with the pGL3-(NR1)5-LUC reporter construct and hGR vector demonstrated that none of the PXR ligands used in this study increased
CYP2B6 reporter gene expression over that of controls in cells expressing hGR alone (Fig. 6C).

**Effects of Cotransfected GR on Human CAR Activation by Different Compounds.** In the absence of exogenous ligand, cell lines transfected with human CAR exhibited high basal reporter activity compared with those without nuclear receptor transfection, which is in agreement with the observations of Sueyoshi et al. (1999). Further treatment with RIF, PB, or CLZ did not significantly increase PBREM reporter activity (Fig. 7A). In addition, the presence of 0.1 μM DEX did not change the CAR-mediated CYP2B6 reporter gene expression pattern under these cotransfection conditions. In contrast, Huh7 cells cotransfected with GR showed markedly enhanced hCAR-mediated reporter activity in the presence of 0.1 μM DEX (Fig. 7B). Under these conditions, treatment with 1 mM PB caused an increase (2-fold) in reporter activity over that of controls, whereas RIF and CLZ elicited no increase in reporter expression.

**Discussion**

Transcriptional regulation of CYP2B gene expression in rodents has been well characterized, and it has been established that CAR predominantly mediates CYP2B1 and CYP2b10 expression in rats and mice, respectively (Honkakoski et al., 1998; Wei et al., 2000). However, notable species-specific differences in CYP2B induction and CAR activation have hampered the extrapolation of these data to humans. Although human CAR exhibits some common characteristics with its rodent counterparts, such as its ability to be translocated into the nucleus upon PB treatment, and the ability of binding to the conserved PBREM sequence, notable differences exist between rodent and human CAR (Pascussi et al., 2000b). For example, androstenediol, progesterone, androgens, and calcium/calmodulin-dependent kinase inhibitors, which are known mCAR inhibitors, do not inhibit activation of hCAR (Zelko and Negishi, 2000). To date, no effective human CAR activator has been reported in transfection assays using cell lines. This is in part due to the constitutive activity of CAR in cell-based transfection assays.

Recently, a number of studies have suggested that the human CYP2B6 gene might be cross-regulated by PXR because it can recognize sequences in the known PBREM located upstream of the CYP2B6 gene (Xie et al., 2000; Goodwin et al., 2001). In this study, we report that a number of known PXR ligands can effectively induce CYP2B6 expression and activity in primary human hepatocyte cultures and that CYP2B6 induction by these ligands is augmented by submicromolar concentrations of DEX. These results indicate that optimal PXR activation of human PBREM occurs through a GR-dependent mechanism.

DEX is an important synthetic glucocorticoid and has been reported to play a biphasic role in CYP3A4 and glutathione S-transferase A2 gene expression (Falkner et al., 2001; Pascussi et al., 2001). Previous studies have indicated that submicromolar concentrations of DEX (≤0.1 μM) enhance PB-induced CYP2B6 expression in primary human hepatocytes (Pascussi et al., 2000b). In the current study, a physiologically relevant concentration of DEX (0.1 μM) was shown to effectively enhance CYP2B6 induction in human hepatocyte cultures treated with various human PXR ligands. Inasmuch as DEX is neither an activator of hCAR nor hPXR at this concentration, an indirect effect of the nuclear receptor GR must be involved in this process.

Using low concentrations of DEX (0.001–1 μM), which have

A. Human primary hepatocytes from HLN-131 were treated with 1 and 10 μM RIF, 100 and 1000 μM PB, 50 μM PHY, or 10 μM CLZ in the absence (lanes 2–7) or presence of 0.1 μM DEX (lanes 8–13), and 0.1% DMSO was used as vehicle control (lane 1). Microsomal protein (30 μg) was analyzed by Western immunoblot with antibodies to CYP2B6 and subjected to densitometric analysis as described under Materials and Methods. B. Microsomal samples (0.1 mg) from hepatocyte cultures after treatment with hPXR ligands in the presence and absence of DEX were analyzed for bupropion hydroxylase (CYP2B6) activity. C. Total RNA extracted from hepatocyte cultures treated as described above was analyzed using real-time PCR for CYP2B6 mRNA abundance.
been shown to activate GR but not PXR (Kliewer et al., 1998; Pascussi et al., 2000a), we found that the constitutive levels of both CYP2B6 protein content and activity were not affected, whereas a dose-dependent induction of CYP3A4 protein content and activity was observed. Recently, El-Sankary et al. (2002) reported that mutation of an HNF-3/CCAAT-enhancer protein binding site in the CYP3A4 proximal promoter could disrupt DEX-mediated CYP3A4 reporter activity, but did not affect induction by the PXR ligand rifampicin. These results indicated that GR could regulate CYP3A4 expression through direct activation of sequences in the CYP3A4 proximal promoter region that is independent of PXR. Moreover, using GR knockout mice, Schuetz et al. (2000) reported that GR is essential for both the basal and DEX-inducible expression of Cyp2b10, suggesting a direct involvement of GR in mouse CYP2B gene expression (Schuetz et al., 2000). Overall, these results indicate that different mechanisms are involved in the regulation of the basal expression of CYP2B6 and CYP3A4 by physiological concentrations of glucocorticoids and that human and mouse GR exert distinct effects on CYP2B gene regulation.

PBREM is highly conserved among the CYP2B gene subfamily
members and is believed to be the most important CYP2B6 regulatory unit (Sueyoshi et al., 1999). Several lines of evidence indicate that PXR cross-regulates CYP2B expression by recognizing this PB response element. In vitro-translated PXR has been shown to effectively bind to the NR1 and NR2 within PBREM located upstream of the CYP2B6 gene using electrophoretic mobility shift assay (Goodwin et

FIG. 6. Activation of CYP2B6 (NR1) reporter gene by hPXR and GR in Huh7 cells.

A, luciferase reporter vector containing five repeats of the PBREM NR1 element was cotransfected with hPXR and pRL-TK in Huh7 cells. HPXR-mediated activation of the reporter gene expression was characterized by treatment of transfected cells with RIF (1 and 10 μM), PB (100 and 1000 μM), PHY (50 μM), CLZ (10 μM), or vehicle (0.1% DMSO), in the presence and absence of 0.1 μM DEX for 24 h before dual luciferase measurement. B, cotransfection of hGR expression vector and pSG5-hPXR with the (NR1) reporter construct and the internal control vector into Huh7 cells as described under Materials and Methods. hPXR ligand (as indicated in A) mediated CYP2B6 reporter gene expression is enhanced by DEX (0.1 μM)-activated hGR in the transfected cells. Data represent the mean ± S.D. of three individual transfections. C, cotransfection of hGR expression vector with the (NR1) reporter construct and the internal control vector into Huh7 cells as described under Materials and Methods. None of the PXR ligands showed activated CYP2B6 reporter gene expression in the presence of the hGR expression vector alone.
In contrast, Huh7 cell-based transfection assays demonstrated that exposure to PXR ligands in the absence of GR cotransfection and DEX cotreatment results only in modest PBREM activation. On the other hand, DEX-activated human GR can greatly enhance PXR-mediated activation of the CYP2B6 reporter gene by various CYP3A4 inducers and PXR ligands.

Currently, the exact mechanism of this DEX effect on CYP2B6 expression is not clear. Because low levels of DEX are required for the maintenance of hepatocyte cultures (Macdonald et al., 2001), one explanation might be that DEX enhances the expression of other genes, including coactivators, such as steroid receptor coactivators or cAMP response element-binding protein, by a nonspecific mechanism that maintains the overall transcriptional and synthetic capacity of the cells (Laishes and Williams, 1976). Although DEX is involved in multiple gene expression patterns and physiological functions, our results in cell-based transfection assays have revealed that DEX-

![Graph A](image1)

**Fig. 7.** Activation of CYP2B6 PBREM by hCAR and hGR in Huh7 cells.

Huh7 cells were transiently transfected with CYP2B6 PBREM reporter vector, pRL-TK vector, and hCAR expression vector in the absence (A) or presence of exogenous hGR expression vector (B). Transfected cells were treated with RIF (10 μM), PB (1000 μM), CLZ (10 μM), or vehicle (0.1% DMSO) in the presence or absence of 0.1 μM DEX for 24 h before dual luciferase measurement. Data represent the mean ± S.D. of three individual transfections.

![Graph B](image2)
mediated GR activation is essential for the enhanced effect on PXR activation of the CYP2B6 protein and gene expression. Neither DEX nor exogenous GR alone could increase PXR-mediated CYP2B6 reporter gene expression. Moreover, these studies have demonstrated that activated GR can also enhance hCAR-mediated constitutive expression of CYP2B6 reporter gene in Huh7 cells. Under this condition, only 1 mM PB treatment caused a significant increase in CYP2B6 reporter gene expression compared with that of controls, whereas RIF and CLZ had no effect on hCAR activation.

Another possible explanation for these synergistic effects is that 0.1 μM DEX increases the expression of PXR and CAR through a potential GRE located in the promoter region of two nuclear receptors (Pascussi et al., 2000b; Maurel, 2002). However, results from this study indicate that additional mechanisms may also be involved in this process. Under the current experimental conditions, 0.1 μM DEX did not significantly increase the expression of human PXR mRNA expression levels. Thus, enhancement of CYP2B6 induction by PXR ligands in the presence of 0.1 μM DEX cannot be fully explained by DEX induction of PXR expression. Moreover, in the Huh7 cell transfection system, the endogenous PXR levels are extremely low and the PXR expression vector lacks the PXR promoter region, which might potentially contain a GRE. This suggests that an increase in PXR expression levels alone cannot account for the observed enhancement of cotransfecting hGR in this system.

An alternative mechanism is that activated GR translocates from the cytoplasm into the nucleus where PXR, XR, and activated CAR are located, and functions as a coactivator to facilitate binding of nuclear receptor heterodimers to the response elements of target genes. Although several studies have indicated that both NR1 and NR2 are important for the optimal activation of the CYP2B2 gene, the role of the NR1 motif plays a much more important role in CYP2B2 regulation (Honkasolo et al., 1998; Goodwin et al., 2001). In this report, we generated a pGL3-NR1(–224)–LUC reporter construct and found similar activation profiles as was observed for the full PRBEM in the PXR/GRE cotransfection assay. These results indicate that PXR-mediated CYP2B6 expression occurs predominantly through PXR activation of the NR1 of PRBEM. Although several GREs have been identified in the regulatory region of rat and mouse CYP2B genes, some of which can be activated by GR (Jaiswal et al., 1990), no functional consensus GRE has been identified in the human CYP2B6 gene thus far. Accordingly, human GR may function as a coactivator to facilitate PXR and CAR binding to their target genes. It remains to be determined whether a potential GRE might exist in the regulatory regions of the PXR or CAR genes, and whether GR is involved directly in the regulation of other nuclear receptors.

In summary, our data suggest that PXR ligands and CYP3A4 inducers can effectively induce human CYP2B6 expression through PXR-mediated PRBEM activation. More importantly, human GR is integrally involved in the regulation of CYP2B6 by both PXR and CAR. These studies also suggest an additional mechanism for the GR-enhanced induction of CYP2B6 by prototypical inducers, whereby activated GR acts as a coactivator to facilitate the binding of PXR and CAR to CYP2B6 response elements. As such, physiological levels of glucocorticoids could differentially influence CYP2B6 induction by various nuclear receptor ligands through a GR-mediated pathway.

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