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ACCELERATED COMMUNICATION

Regulation of the Human *CYP2B6* Gene by the Nuclear Pregnane X Receptor

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ABSTRACT

Cytochromes P450 (P450s) are involved in the oxidative metabolism of a plethora of structurally unrelated compounds, including therapeutic drugs. Two orphan members of the nuclear receptor superfamily, the pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3) have been implicated in this phenomenon. In the present study, we examined the transcriptional regulation of the human *CYP2B6* gene. In primary cultures of human hepatocytes, *CYP2B6* was highly inducible by a number of compounds known to be human PXR ligands, including rifampicin and hyperforin. PXR was shown to be capable of activating the phenobarbital-responsive enhancer module (PBREM) region of the *CYP2B6* gene, a 51-base-pair enhancer element that mediates induction of *CYP2B6* expression by CAR. The two nuclear receptor-binding motifs within the PBREM effectively bound PXR as a heterodimer with the 9-*cis* retinoic acid receptor α (NR2B1). Taken together, these observations demonstrate that the *CYP2B6* gene is directly regulated by PXR and further establish this receptor as a key regulator of drug-metabolizing P450s.

Cytochromes P450 (P450s) are a superfamily of hemethiolate–containing proteins involved in the oxidative metabolism of a diverse range of compounds, including steroid hormones, bile acids, fatty acids, and prostaglandins. In addition, many P450 enzymes participate in the conversion of carcinogens, environmental pollutants, and drugs to more polar metabolites, thereby facilitating their excretion and preventing the accumulation of these potentially harmful compounds (Nelson et al., 1996).

For many years, it has been understood that xenobiotic compounds can induce the expression of certain P450 genes, notably members of the *CYP1A*, *CYP2B*, *CYP3A*, and *CYP4A* subfamilies (Waxman, 1999). This adaptive response increases the organism's ability to metabolize and ultimately eliminate toxic and carcinogenic compounds. Whereas induction of *CYP1A* genes by aromatic hydrocarbons and *CYP4A* subfamily members by peroxisome proliferators are known to be mediated by the aryl hydrocarbon receptor and peroxisome proliferator activated receptor α (NR1C1), respectively, the molecular mechanisms by which structurally dissimilar compounds induce *CYP2B* and *CYP3A* genes remained obscure (Denison and Whitlock, 1995). Recently, a number of laboratories identified two orphan members of the nuclear receptor family, the pregnane X receptor (PXR; NR1I2) and constitutive androstane receptor (CAR; NR1I3), as xenobiotic-responsive transcription factors (Bertilsson et al., 1998; Blumberg et al., 1998; Honkakoski et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Moore et al., 2000b; Tzameli et al., 2000)

Human PXR (also known as SXR or PAR) binds and is activated by many known *CYP3A4* inducers, including the macrocyclic antibiotic rifampicin, the antimycotic clotrimazole, the barbiturate phenobarbital (PB), and the putative antidepressant component of St. John's wort, hyperforin (Bertilsson et al., 1998; Blumberg et al., 1998; Lehmann et al., 1998; Jones et al., 2000; Moore et al., 2000a). PXR interacts with its cognate response elements in the 5′-flanking regions of *CYP3A* genes as a heterodimer with the 9-*cis* retinoic acid receptor α (RXRα; NR2B1). Typically, these elements contain two copies of the AG(G/T)TCA hexad organized as a direct repeat with a three-nucleotide spacer (DR3) or an everted repeat (ER) separated by 6 bp (ER6) (Bertilsson

ABBREVIATIONS: P450; cytochrome P450, PXR; pregnane X receptor; CAR; constitutive androstane receptor; RXRα; 9-*cis* retinoic acid receptor α; DR*n*; direct repeat with *n*-bp spacer; bp, base pair(s); ER6; everted repeat with 6-base-pair spacer; PB; phenobarbital; PBRU, phenobarbital-responsive unit; PBREM; phenobarbital-responsive enhancer module; FBS, fetal bovine serum; DMSO, dimethyl sulfoxide; SPAP, secreted placental alkaline phosphatase; EMSA, electrophoretic mobility-shift assay.

et al., 1998; Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999). Further compelling evidence for the role of PXR in the induction of *CYP3A* genes is provided by experiments performed in mice harboring a homozygous disruption of the *pxr* gene. Thus, mice lacking functional PXR fail to up-regulate *Cyp3a11* expression in response to the classic rodent *CYP3A* inducers pregnenolone 16α-carbonitrile and dexamethasone (Xie et al., 2000a; Staudinger et al., 2001). Notably, induction of *Cyp3a11* expression by PB was intact in the PXR-null mice (Xie et al., 2000b; Staudinger et al., 2001).

In addition to PXR, CAR also plays a central role in the regulation of xenobiotic-inducible P450 genes, although the biology of this receptor is very different from that of PXR. CAR exhibits a high level of constitutive transcriptional activity and can activate expression of reporter gene constructs in the absence of exogenously added ligand (Baes et al., 1994; Choi et al., 1997). The androstane metabolites androstanol and androstenol act as inverse agonists for the mouse and, to a lesser extent, human CAR, whereas the potent *Cyp2b10* inducer 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene is a high-affinity mouse CAR agonist (Forman et al., 1998; Moore et al., 2000b; Tzameli et al., 2000). As with PXR, there seems to be a species divergence in CAR pharmacology (Jones et al., 2000; Moore et al., 2000b).

Elegant studies by Negishi and coworkers have demonstrated that induction of *CYP2B* subfamily members by PB and PB-like inducers is mediated by CAR (Honkakoski et al., 1998; Kawamoto et al., 1999). In untreated liver, CAR resides in the cytoplasm of the hepatocyte. However, exposure of the cell to PB or PB-like inducers promotes the rapid translocation of CAR to the nucleus, where it *trans*-activates expression of its target genes. Importantly, this process is uncoupled by the phosphatase inhibitor okadaic acid, suggesting that the PB-induced nuclear translocation of CAR is a phosphorylation-sensitive event (Kawamoto et al., 1999). Although PB does not seem to interact directly with CAR (Moore et al., 2000b), targeted disruption of the mouse *car* gene results in total ablation of *Cyp2b10* inducibility by both PB and 1,4-bis[2-(3,5-dichloropyridyloxy)]benzene (Wei et al., 2000). After translocating to the nucleus, CAR interacts with a conserved 51-bp enhancer element located ~2 kilobase pairs upstream of the *CYP2B1*, *CYP2B2*, *CYP2B6*, and *Cyp2b10* transcription initiation sites (Trottier et al., 1995; Honkakoski et al., 1998; Sueyoshi et al., 1999; Smirlis et al., 2001). These regions, termed phenobarbital-responsive units (PBRU) or phenobarbital-responsive enhancer modules (PBREM), contain two DR4 elements (NR1 and NR2) that act as high-affinity binding sites for CAR and its obligate heterodimerization partner RXRα. Notably, PXR is also reported to *trans*-activate DR4 elements; moreover, PXR and CAR bind and activate common response elements in the human *CYP3A4* and rodent *CYP3A23*, *CYP2B1*, and *Cyp2b10* genes, suggesting that interplay between these two receptors is likely to be a central theme in the regulation of xenobiotic-inducible P450s (Blumberg et al., 1998; Sueyoshi et al., 1999; Xie et al., 2000b; Geick et al., 2001; Smirlis et al., 2001; B. Goodwin, E. Hodgson, and C. Liddle, submitted).

In this study, we examined the role of PXR in the regulation of the human *CYP2B6* gene. CYP2B6 is involved in the metabolism of a number of clinically important drugs (Ekins and Wrighton 1999); moreover, its expression is reported to be induced by compounds that are PXR ligands, including rifampicin, PB, troglitazone, and dexamethasone (Strom et al., 1996; Chang et al., 1997; Sahi et al., 2000; Gerbal-Chaloin et al., 2001). We show that PXR directly regulates *CYP2B6* expression.

Experimental Procedures

Materials. Rifampicin, dexamethasone, sodium phenobarbital, and charcoal-stripped, delipidated FBS were obtained from the Sigma Chemical Co. (St. Louis MO). Cell culture reagents, unless otherwise stated, were provided by Invitrogen (Carlsbad, CA). Hyperforin was purchased from Apin Chemicals Ltd. (Abingdon, Oxon, UK). SR12813 was synthesized in house.

Primary Culture of Human Hepatocytes and Northern Blot Analysis. Primary human hepatocytes were obtained from Dr. Stephen Strom (University of Pittsburgh, Pittsburgh, PA) and maintained exactly as described elsewhere (Moore et al., 2000a). At 48 h after isolation, cells were treated for a further 48 h with various inducers that were added to the culture medium as 1000× stocks in DMSO. Sodium phenobarbital was dissolved directly into the medium. Control cultures received vehicle (0.1% DMSO) alone. Total RNA was isolated using a commercially available reagent (TriZOL; Invitrogen). CYP2B6 and CYP3A4 mRNA levels were examined by Northern blot analysis using standard techniques. Blots were sequentially hybridized with CYP2B6 [bases 3–659 of the published cDNA; GenBank accession number AF182277), CYP3A4 (bases 790 to 1322 of the published cDNA; GenBank accession number M18907), and β-actin (CLONTECH Laboratories Inc., Palo Alto, CA) cDNA probes.

Preparation of *CYP2B6* PBREM Reporter Gene Constructs. Luciferase reporter gene constructs were prepared by annealing oligonucleotides corresponding to the wild-type and mutant *CYP2B6* PBREM (Fig. 2A) before insertion into the *Bgl*II site of pGL3-tk-Luc, which contains bases −105 to +51 of the herpes simplex virus thymidine kinase promoter linked to a luciferase reporter gene.

Transient Transfection Assays. Analysis of PXR- and CAR-dependent *trans*-activation of the *CYP2B6* PBREM reporter gene constructs was performed in a human liver-derived cell line, HuH7. Cells (20,000 per well) were inoculated into a 96-well plate in Dulbecco's modified Eagle's/Ham's F12 media nutrient mixture supplemented with 10% charcoal/dextran-treated FBS (HyClone Laboratories Inc., Logan, UT) and transfected 24 h later with LipofectAMINE Plus reagent (Invitrogen). Transfection mixes contained 8 ng of luciferase reporter gene construct, 2 ng of human CAR or PXR expression vectors, pSG5-hCAR (Moore et al., 2000b) and pSG5-ΔATG-hPXR (Lehmann et al., 1998), 8 ng of pβ-actin-SPAP, and 52 ng pBluescript (Stratagene, La Jolla, CA). Transfection was allowed to proceed for 3 h. Cells were maintained for a further 24 h in the presence of drug (added as a 1000× stock in DMSO) in Dulbecco's modified Eagle/Ham's F12 media nutrient mixture supplemented with 10% heat-inactivated, charcoal-stripped, delipidated FBS. An aliquot of medium was withdrawn for SPAP assay and the cells lysed before luciferase determination. Luciferase activities were normalized to SPAP expression.

Electrophoretic Mobility-Shift Assay. Electrophoretic mobility-shift assay (EMSA) was performed exactly as described elsewhere (Goodwin et al., 1999). In vitro translated human RXRα, CAR, and PXR were prepared using a TNT rabbit reticulocyte system (Promega, Madison, WI). Binding reactions were preincubated on ice for 10 min before the addition of ^{32}P-end-labeled probe corresponding to the NR1 and NR2 motifs. After a further 20 min on ice, samples were resolved on a pre-electrophoresed 5% acrylamide gel in 0.25× Tris/borate/EDTA buffer (22.5 mM Tris-borate, 0.5 mM EDTA).

Results

Induction of *CYP2B6* Expression by PXR Ligands. To examine whether PXR regulates *CYP2B6* expression, human hepatocytes were treated with a panel of known PXR ligands (Fig. 1A). In line with earlier observations, rifampicin (~15-fold) and PB (~50-fold) effectively induced CYP2B6 mRNA levels (Strom et al., 1996; Chang et al., 1997; Gervot et al., 1999; Gerbal-Chaloin et al., 2001). In addition, treatment of human hepatocytes with dexamethasone or the high-affinity PXR ligands SR12813 and hyperforin (Jones et al., 2000; Moore et al., 2000a) resulted in the induction of CYP2B6 expression (~6-fold, 5-fold, and 4-fold, respectively). In parallel with CYP2B6 mRNA levels, expression of CYP3A4 was also examined. As expected, CYP3A4 mRNA levels were strongly induced by rifampicin (13-fold), SR12813 (~5-fold), hyperforin (~3.5-fold), dexamethasone (7-fold), and PB (~13-fold) (Fig. 1B). Similarly, rifampicin, SR12813, hyperforin, and dexamethasone induced *CYP2B6* and *CYP3A4* expression. However, the PB-mediated induction of *CYP2B6* (~50-fold) expression was significantly higher than that of *CYP3A4* (~13-fold), in line with the important role of CAR in the regulation of *CYP2B6* (Sueyoshi et al., 1999).

PXR Activates the *CYP2B6* PBREM. Transcriptional activation of the *CYP2B6* gene by CAR is mediated by the 51-bp PBREM. This region is located 1.7 kilobase pairs upstream of the *CYP2B6* transcription initiation site and contains two imperfect DR4 elements, designated NR1 and NR2 (Fig. 2A) (Sueyoshi et al., 1999). Importantly, PXR-RXRα heterodimers are reported to be capable of binding and *trans*-activating DR4 elements (Blumberg et al., 1998). Taken together, these observations suggested that induction of *CYP2B6* expression by compounds that activate PXR may be mediated by the PBREM region. Thus, we examined the ability of PXR to activate reporter gene constructs harboring the *CYP2B6* PBREM linked to a minimal herpes simplex virus thymidine kinase promoter and luciferase reporter. Chimeric *CYP2B6*-PBREM reporter gene constructs containing wild-type and mutated DR4 motifs (Fig. 2A) were transiently transfected into a liver-derived cell line (HuH7) and the ability of rifampicin, a human PXR ligand, to activate expression of these constructs was determined. In the absence of exogenously expressed PXR, there was no detectable induction of reporter gene expression by rifampicin (Fig. 2B). Cotransfection of the wild-type *CYP2B6*-PBREM construct (pGL3-CYP2B6-PBREM) with a human PXR expression vector resulted in an ~2-fold increase in reporter gene activity. Treatment of transfected cells with rifampicin (10 μM) elicited a further 3-fold induction of luciferase expression (Fig. 2B). The relative contribution of the NR1 and NR2 DR4 motifs to the PXR response was examined by mutating the hexad half-sites in one or both of these elements. Although mutation of the NR1 site (pGL3-CYP2B6-PBREMmutNR1)

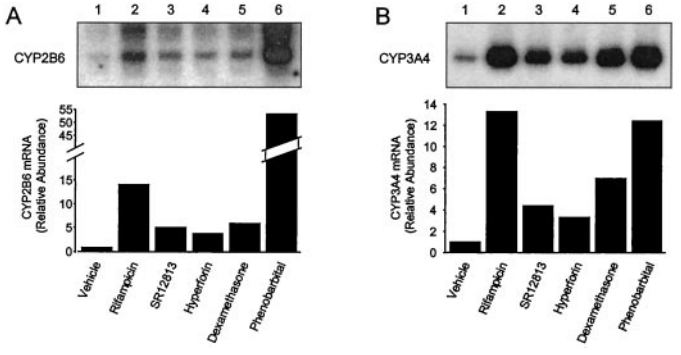

Fig. 1. Induction of *CYP2B6* and *CYP3A4* expression in primary cultures of human hepatocytes. Northern blot analysis of CYP2B6 (A) and CYP3A4 (B) mRNA levels was performed with total RNA (10 μg) prepared from human hepatocytes treated for 48 h with vehicle alone (0.1% DMSO; lane 1), rifampicin (10 μM; lane 2), SR12813 (1 μM; lane 3), hyperforin (10 μM; lane 4), dexamethasone (10 μM; lane 5), or PB (1 mM; lane 6). Relative mRNA abundance (corrected for β-actin expression) is depicted graphically at the bottom of each panel.

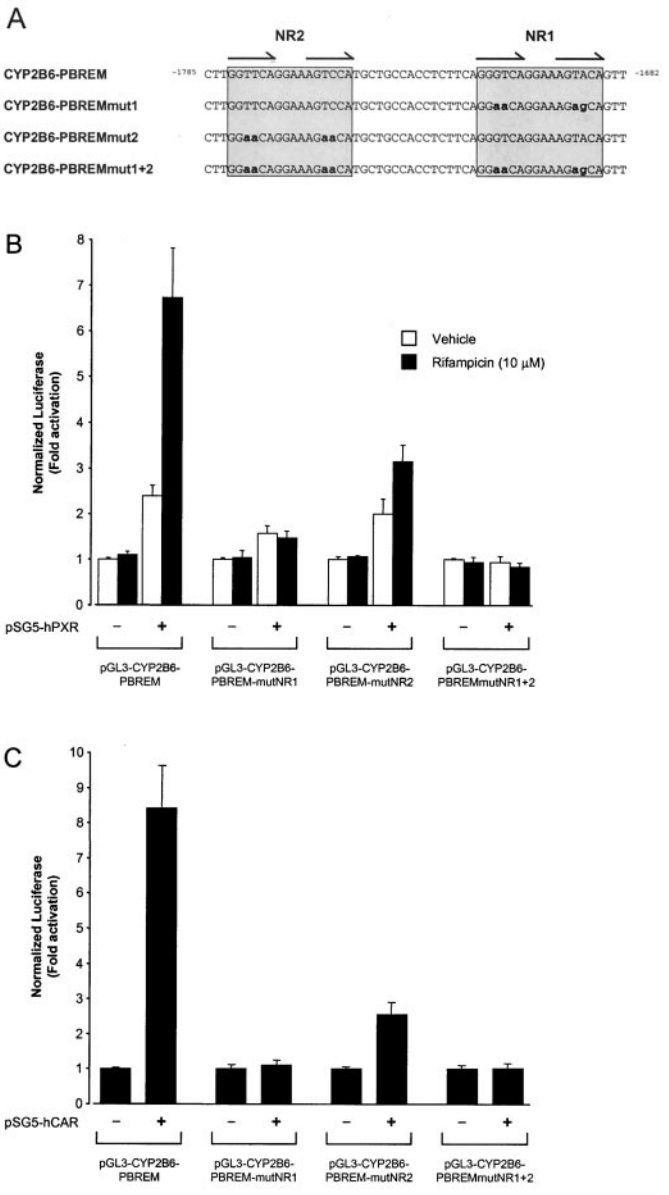

Fig. 2. Activation of the *CYP2B6* PBREM by PXR and CAR. A, structure of the *CYP2B6* PBREM. The two DR-4 elements (NR1 and NR2) are delineated by shaded boxes. The repeated half-sites within these elements are indicated by arrows. The mutated bases in the pGL3-CYP2B6-PBREMmut1, pGL3-CYP2B6-PBREMmut2, and pGL3-CYP2B6-PBREMmut1 + 2 are shown in lower case. The position relative to the *CYP2B6* transcription initiation site is also shown. *trans*-Activation of the wild-type and mutant *CYP2B6* PBREM constructs by PXR (B) and CAR (C) was examined by transient transfection in HuH7 cells as described under *Experimental Procedures*. PXR-mediated activation of the *CYP2B6* PBREM cells was characterized by treatment of transfected cells with rifampicin (10 μM) or vehicle (0.1% DMSO) for 24 h prior to harvest. Data represent the mean ± S.D. of four individual transfections.

completely abrogated PXR-dependent activation, the pGL3-CYP2B6-PBREMmutNR2 construct, which contains a mutated NR2 element, retained some responsiveness to PXR. As expected, mutation of both the NR1 and NR2 sites (pGL3-CYP2B6-PBREMmut1 + 2) destroyed the PXR response (Fig. 2B). These data suggest that the NR1 motif is quantitatively the more important element to the PXR response.

In parallel transfection experiments, CAR effectively activated (~8-fold) the wild-type *CYP2B6* PBREM reporter (Fig. 2C). Disruption of the NR1 site caused a total loss in CAR activation; mutation of the NR-2 resulted in a substantial reduction in the CAR-dependent reporter activity. However, some residual CAR-responsiveness remained (Fig. 2C). The relative importance of the NR1 element in the CAR response is in agreement with earlier reports by Negishi and colleagues (Sueyoshi et al., 1999).

The NR1 and NR2 Motifs Bind PXR-RXRα Heterodimers. The ability of the NR1 and NR2 sites to bind PXR was examined by EMSA. Both the NR1 and NR2 elements strongly complexed PXR-RXRα heterodimers (Fig. 3A). In close agreement with the cell-based reporter gene assays described above, competition binding studies demonstrated that the NR1 site bound PXR-RXRα with higher affinity than the NR2 motif (Fig. 3B), confirming that this site is the predominant PXR response element within the PBREM. As reported previously (Sueyoshi et al., 1999), CAR-RXRα heterodimers bound both NR1 and NR2 (Fig. 3A). In similarity to the PXR-RXRα binding profile, the NR1 site interacted with the CAR-RXRα heteromer with significantly higher affinity than the NR2 element (Fig. 3B). The mutated derivatives of the NR1 and NR2 sites used in the transient transfection studies failed to compete for either PXR-RXRα or CAR-RXRα binding.

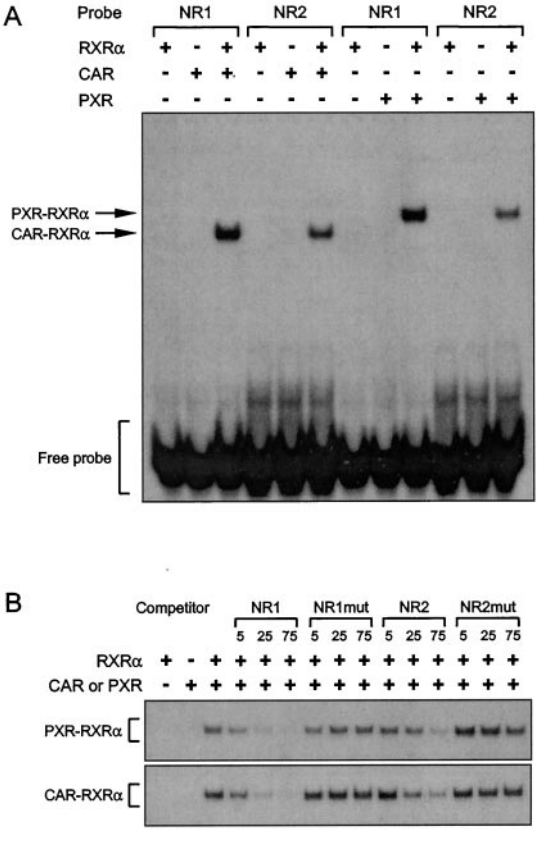

Fig. 3. PXR binding to the NR1 and NR2 response elements. The ability of CAR-RXRα and PXR-RXRα-heterodimers to bind the DR4 elements (NR1 and NR2) in the *CYP2B6* PBREM was examined by EMSA as outlined under *Experimental Procedures*. A, EMSA with radiolabeled double-stranded oligonucleotides corresponding to the NR1 and NR2-binding motifs (see Fig. 2A). Reactions received in vitro translated RXRα, CAR, and PXR as indicated. B, competition EMSA was performed using the NR1 motif as probe. Unlabeled competitor oligonucleotides corresponding to the wild-type and mutant NR1 and NR2 sites (see Fig. 2A) were added to the preincubation reaction at the indicated molar excess.

Discussion

A number of earlier studies suggested that the human *CYP2B6* gene might be regulated in a PXR-dependent manner (Ekins and Wrighton 1999, and references therein). In this report, we show that *CYP2B6* is regulated directly by PXR. In primary cultures of human hepatocytes, the human PXR ligands rifampicin, hyperforin, SR12813, dexamethasone, and PB effectively induced expression of both *CYP2B6* and *CYP3A4*, a well-documented PXR target gene. *Trans*-activation of *CYP2B6* by PXR was shown to be mediated by the PBREM region of the gene, a 51-bp enhancer module that controls induction of *CYP2B6* by CAR (Sueyoshi et al., 1999).

The PBREM/PBRU is highly conserved among PB-inducible *CYP2B* subfamily members, namely *CYP2B1*, *CYP2B2*, *CYP2B6*, and *Cyp2b10* (Sueyoshi et al., 1999); moreover, similar to *CYP2B6*, both the mouse *Cyp2b10* PBREM and rat *CYP2B1* PBRU are activated by PXR (Xie et al., 2000b; Smirlis et al., 2001). The *CYP2B6* PBREM contains two DR4 elements that are capable of binding both PXR-RXRα and CAR-RXRα heteromers. CAR was originally reported to bind a DR5 element in the RARβ2 promoter; subsequently, however, CAR-mediated *trans*-activation through DR3, DR4, and ER6 elements has been documented (Baes et al., 1994; Choi et al., 1997; Honkakoski et al., 1998; Sueyoshi et al., 1999; Xie et al., 2000; Smirlis et al., 2001; B. Goodwin, E. Hodgson, and C. Liddle, submitted). It is now apparent that these configurations of nuclear receptor half-sites are also capable of binding PXR-RXRα heterodimers (Blumberg et al., 1998; Kliewer et al., 1998; Lehmann et al., 1998; Goodwin et al., 1999; Sueyoshi et al., 1999; Xie et al., 2000b; Geick et al., 2001; Smirlis et al., 2001). Taken together, these observations demonstrate that CAR and PXR are capable of regulating common genes through the same *cis*-acting elements, suggesting that cross talk between these two signaling pathways is an important factor in mounting an appropriate response to a xenobiotic challenge.

Although rifampicin and PB induced *CYP3A4* to a similar extent (~13-fold), *CYP2B6* was substantially more responsive to PB (~50-fold) than rifampicin (~15-fold). Thus, although both CAR and PXR directly regulate *CYP2B6* expression, CAR seems to assume a dominant role in the PB-mediated induction of this gene. It is possible that the arrangement of the nuclear receptor binding motifs within the *CYP2B6* PBREM provides an optimal platform for CAR-mediated transactivation. In addition to promoting nuclear translocation of CAR, PB is an effective activator of human PXR (Lehmann et al., 1998; Goodwin et al., 1999; Jones et al., 2000). We have previously shown that CAR and PXR share common ligands (Moore et al., 2000b); therefore, it is likely that certain xenobiotics, including PB, are capable of inducing P450 expression through multiple signaling pathways.

The existence of multiple xenobiotic receptors with distinct but overlapping ligand specificities increases the organism's ability to detect and respond to a potentially harmful substance.

In summary, we have shown that the human *CYP2B6* gene is directly regulated by PXR. These results provide evidence for a functional redundancy between the nuclear receptors PXR and CAR in the protective response to xenobiotic challenge in humans. Importantly, our findings extend the range of potential drug interactions caused by compounds that activate PXR to include CYP2B6 substrates. This knowledge can be used to understand more fully the metabolism of drugs currently on the market and to design safer drugs for the future.

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