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Environmental Xenobiotics and the Antihormones Cyproterone Acetate and Spironolactone Use the Nuclear Hormone Pregnenolone X Receptor to Activate the *CYP3A23* Hormone Response Element

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ABSTRACT

The pregnenolone X receptor (PXR), a new member of the nuclear hormone receptor superfamily, was recently demonstrated to mediate glucocorticoid agonist and antagonist activation of a hormone response element spaced by three nucleotides (DR-3) within the rat *CYP3A23* promoter. Because many other steroids and xenobiotics can up-regulate CYP3A23 expression, we determined whether some of these other regulators used PXR to activate the *CYP3A23* DR-3. Transient cotransfection of LLC-PK1 cells with *(CYP3A23)$_2$-tk-CAT and mouse PXR demonstrated that the organochlorine pesticides transnonachlor and chlordane and the nonplanar polychlorinated biphenyls (PCBs) each induced the CYP3A23* DR-3 element, and this activation required PXR. Additionally, this study found that PXR is activated to induce (*CYP3A23*)$_2$-tk-CAT by antihormones of several steroid classes including the antimineralocorticoid spironolactone and the antiandrogen cyproterone acetate. These studies reveal that PXR is involved in the induction of CYP3A23 by pharmacologically and structurally distinct steroids and xenobiotics. Moreover, PXR-mediated PCB activation of the (*CYP3A23*)$_2$-tk-CAT may serve as a rapid assay for effects of nonplanar PCBs.

The human and rat CYP3A forms are induced by numerous steroidal hormones; a hallmark feature is their nonclassical induction by glucocorticoids (Schuetz and Guzelian, 1984; Schuetz *et al.*, 1984). Characteristics of this nonclassical induction include requirements for pharmacological amounts of steroid to induce CYP3A as well as the paradoxical induction of CYP3A23[1] by both the glucocorticoid agonist dexamethasone and the antiglucocorticoids PCN (Schuetz *et al.*, 1984) and RU486 (Kocarek *et al.*, 1995). We previously hypothesized that other classes of antihormones might share regulatory characteristics and induce CYP3A as well (Kocarek *et al.*, 1995). Further studies revealed that antihormone representatives of many of the major steroid classes, including the antimineralocorticoid spironolactone and the antiandrogen cyproterone acetate, were each able to induce hepatic CYPA3A in rat and human hepatocytes (Kocarek *et al.*, 1995) and led us to speculate about a common regulatory factor (Kocarek *et al.*, 1995). Additionally, CYP3A23 is also induced by important environmental xenobiotics including organochlorine pesticides (e.g., transnonachlor and chlordane), and some polychlorinated biphenyls, particularly those with ortho-chlorines (Schuetz *et al.*, 1986).

A new member of the nuclear hormone receptor superfamily, the PXR that mediates glucocorticoid agonist and glucocorticoid antagonist induction of the *CYP3A23* gene has recently been identified (Kliewer *et al.*, 1998). The glucocorticoid agonist or antagonist activated PXR:RXR heterodimer bound to a hormone response element, an AGTTCAtgaAGTTCA direct repeat with a three-nucleotide spacer (DR-3), in the 5′-flanking region of the *CYP3A23* gene to induce its transcription. A number of other hormones (e.g., pregnenolone and progesterone) were also identified as activators of PXR. We have tested the hypothesis that other classes of steroidal antihormones and environmental chemicals might represent additional activators of PXR. We found that transcriptional activation of the *CYP3A23* DR-3 hormone re-

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[1] CYP3A23 refers to the major dexamethasone, PCN and phenobarbital inducible form of CYP3A in rat liver (Komori and Oda, 1994) which is now recognized to be CYP3A23, not CYP3A1. The *CYP3A* 5′flanking regulatory sequence previously identified as *CYP3A1* (Burger *et al.*, 1992; Quattrochi *et al.*, 1995; Kliewer *et al.*, 1998) is in fact *CYP3A23* (Nelson *et al.*, 1993; Barwick *et al.*, 1996). Likewise, because the *CYP3A1* cDNA probe used in all previous publications of CYP3A regulation cannot distinguish between CYP3A1 and CYP3A23, we refer to the CYP3A in these publications as CYP3A23.

ABBREVIATIONS: PCN, pregnenolone 16α-carbonitrile; PXR, pregnane X receptor (mouse PXR.1), GenBank accession no. AF031814; PCB, polychlorinated biphenyl; CAT, chloramphenicol acetyltransferase; RXR, retinoid X receptor.

sponse element by many of these structurally diverse compounds is mediated by a single PXR induction process.

Experimental Procedures

Materials. *(CYP3A23)*$_2$-tk-CAT and mouse PXR.1 (hereafter referred to as PXR) were kindly provided by Dr. Steven Kliewer (Glaxo Wellcome Research and Development, Research Triangle Park, NC). Dexamethasone-*t*-butylacetate was from Research Plus (Bayonne, NJ), organochlorine pesticides from Velsicol (Chicago, IL), and PCBs were given by Stephen Safe (Texas A & M, College Station, TX).

Cell culture. LLC-PK1 pig kidney epithelial cells were obtained from American Type Culture Collection (Rockville, MD) and cultured in medium 199 (Life Technologies, Gaithersburg, MD) containing 10% fetal calf serum.

Transfection assays. LLC-PK1 cells were plated in 24-well dishes at 2.0×10^5 cells per well. Twenty-four hours later, medium was changed and cells transfected with 200 ng of *(CYP3A23)*$_2$-tk-CAT, 66 ng of mPXR and 300 ng of TK-Luciferase by calcium phosphate overnight. The next day, cells were washed once with medium and refed with medium containing 10% charcoal-stripped delipidated calf serum (Sigma) and xenobiotics or steroids. All steroids and xenobiotics were dissolved in dimethyl sulfoxide, with the dimethyl concentration in medium not exceeding 0.1%. Twenty-four hours later, cells were washed once with phosphate-buffered saline and lysed in 100 μl of 1$\times$ luciferase lysis buffer according to manufacturer's instructions (Promega); 35 μl was assayed for luciferase activity and 35 μl was assayed for CAT activity as previously described (Burger *et al.*, 1992). Transfections containing *(CYP3A23)*$_2$-tk-CAT were normalized to TK-luciferase activities. In some cases, BioRad (Richmond, CA) protein assays were performed on 10 μl of remaining lysate according to the manufacturer's instructions.

Results

LLC-PK1 cells were chosen for transfection studies because multiple derivative LLC-PK1 cell lines stably expressing a variety of drug and steroid efflux transporters have been made (Schinkel *et al.*, 1995; Evers *et al.*, 1996). Therefore, if LLC-PK1 cells proved suitable for these transfection studies of PXR, the parent and derivative cell lines could be used in future experiments examining the influence of these transporters on PXR activation. Immunoblot analysis of LLC-PK1 cell lysates with anti-RXR IgG (Affinity Bioreagents) revealed immunodetectable RXR (data not shown). Hybridizable PXR mRNA transcripts were only detectable in lysates of LLC-PK1 cells transfected with the PXR expression vector, but not lysates of nontransfected LLC-PK1 cells (data not shown). We first determined whether PXR could induce the *CYP3A23* DR-3 in LLC-PK1 cells treated with established PXR ligands (Kliewer *et al.*, 1998). Similar to findings in CV-1 cells (Kliewer *et al.*, 1998), the *CYP3A23* DR-3 was induced in LLC-PK1 cells co-transfected with PXR and treated with either dexamethasone-*t*-butylacetate, RU486, progesterone, 1,16-dimethylpregnenolone, pregnenolone, and 5β-pregnane-3,20-dione (Fig. 1), although the fold-increase was not quite as robust as in CV-1 cells. *(CYP3A23)*$_2$-tk-CAT was not transcriptionally activated by these steroids in the absence of co-transfected PXR (data not shown), thus demonstrating PXR's essential role in transactivation by these agents and confirming the original results in CV-1 cells (Kliewer *et al.*, 1998). Moreover, because neither PXR nor RXR alone can bind to the *CYP3A23* DR-3 alone (Kliewer *et al.*, 1998), but only binds as a heterodimer, the transcriptional activation of the *CYP3A23* DR-3 in LLC-PK1 cells further confirms the presence of RXR.

We first tested whether PCBs, previously shown to induce CYP3A23 in rat liver in vivo and in primary rat hepatocytes (Schuetz *et al.*, 1986), could activate *(CYP3A23)*$_2$-tk-CAT in LLC-PK1 cells co-transfected with PXR. Treatment with various nonplanar polychlorinated biphenyls caused dose-dependent activation of *(CYP3A23)*$_2$-tk-CAT (Fig. 2), but only in cells co-transfected with PXR (Fig. 4). Strikingly, except for PCB #47, the rank order of PCB congeners as inducers of *(CYP3A23)*$_2$-tk-CAT was directly associated with the extent of ortho-chlorination, a finding we had shown previously to be correlated with their hierarchy as inducers of the endogenous *CYP3A23* gene in rat hepatocyte cultures (Schuetz *et al.*, 1986). Treatment with 10 μM (data not shown) or 20 μM of another class of environmental contaminants, the organochlorine pesticides transnonachlor or chlordane (Fig. 4) induced the *CYP3A23* DR-3, but only in cells co-transfected with PXR (Fig. 4).

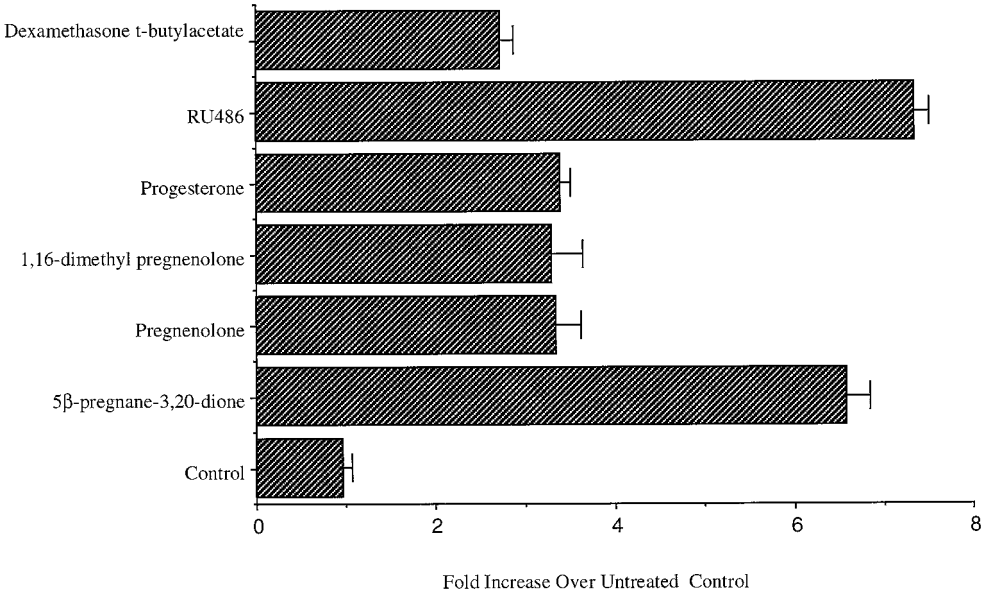

Fig. 1. Steroids activate the *CYP3A23* DR-3 in LLC-PK1 cells co-transfected with PXR. LLC-PK1 cells co-transfected with mouse PXR, *(CYP3A23)*$_2$-tk-CAT, and TK-LUC were treated with 50 μM of the indicated steroids (*control*, no steroid treatment) for 24 hr and cell extracts were assayed for CAT and Luciferase activities. Data was graphed as -fold increase over untreated control and represented CAT activity in treated cells normalized to co-transfected TK-LUC in the same sample; this treatment (CAT/LUC) value was divided by the mean (CAT/LUC) value from 3–4 control wells. The control value was 0.974 ($\pm$) 0.087. The values represent means $\pm$ standard deviation from a representative experiment (repeated two to four times) with three to four replicates per treatment per experiment.

We next determined whether other classes of antihormones besides antiglucocorticoids could use PXR to activate the *CYP3A23* DR-3. The antiandrogen cyproterone acetate dose-response curve for *CYP3A23* DR-3 activation was similar in potency and efficacy to the glucocorticoid agonist dexamethasone-*t*-butylacetate (Fig. 3), whereas the antimineralocorticoid spironolactone was a less efficacious inducer.

For each of the agents not investigated previously (Kliewer *et al.*, 1998) for PXR activation, we compared the activity of *(CYP3A23)*$_2$-tk-CAT in LLC-PK1 cells with or without co-transfected PXR. None of the agents transcriptionally activated *(CYP3A23)*$_2$-tk-CAT in the absence of co-transfected PXR (Fig. 4) or in the presence of co-transfected pSG5 expression vector (Stratagene, LaJolla, CA) substituted for mPXR (data not shown). Importantly, by transfecting cells at a high density (2×10^5/well), we minimized toxic effects of any of the xenobiotics or steroids on cell viability as judged by the recovery of protein per well and trypan blue exclusion. To further control for possible nonspecific effects on the TK promoter, we normalized CAT activity to co-transfected TK-LUC in all experiments (Figs. 1–4). Because the amount of co-transfected hormone receptor can affect the extent of gene activation (data not shown) we tested the possibility that some of the drugs might produce an increase in PXR expression by activating the simian virus 40 promoter driving PXR expression. However, none of the drugs activated simian virus 40-LUC (PGL2-promoter, Promega, Madison, WI) transfected into LLC-PK1 cells (data not shown). Thus, it is unlikely the level of expressed PXR is affected by these drugs.

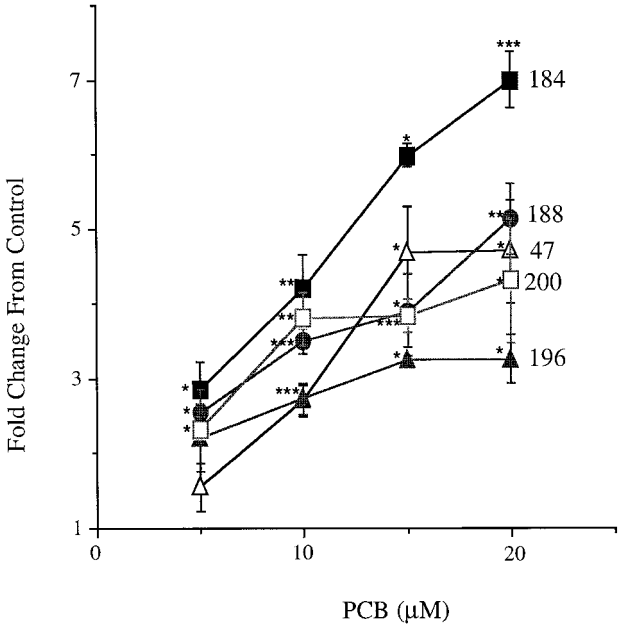

Fig. 2. Polychlorinated biphenyls activate the *CYP3A23* DR-3. LLC-PK1 cells co-transfected as in 1 were treated with 5–20 μM of the indicated PCBs with the position of chlorines as follows: (PCB 184 [■], 2, 2′, 3′, 4, 4′, 6, 6′), (PCB 188 [●], 2, 2′, 3′, 4, 5′, 6, 6′), (PCB 200 [□], 2, 2′, 3, 3′, 4′, 5, 6, 6′), (PCB 196 [▲], 2, 2′, 3, 3′, 4, 4′, 5′, 6), (PCB 47 [△], 2, 2′, 4, 4′). Data was graphed and analyzed as in Figure 1. *Control*, cells co-transfected with *(CYP3A23)*$_2$-tk-CAT, TK-LUC, and PXR in the absence of xenobiotic treatment. The control value was 0.998 ± 0.07. The student's *t* test was used to determine that of the treated values, congener, #47, #196 were not significant at 5 μM with $p < 0.05$ as the limit of significance. All other congeners were significantly different from the control with $p < 0.05$ (*), $p < 0.01$ (**), or $p < 0.005$ (***).

Discussion

More than 15 years have passed since it was determined that organochlorine pesticides and PCBs induce CYP3A (Schuetz *et al.*, 1986). However, the mechanism by which these environmental contaminants up-regulate hepatic *CYP3A* genes has remained elusive. We had demonstrated previously that these agents induce *de novo* synthesis of CYP3A23 in primary rat hepatocyte cultures (Schuetz *et al.*, 1986) and postulated that, like dexamethasone and PCN, these agents might induce CYP3A23 synthesis by interacting with the "PCN receptor" (Schuetz *et al.*, 1986). Our current study identifies PXR, the recently identified receptor activated by PCN, as indispensable in mediating transcriptional activation of the *CYP3A23* DR-3 by environmental contaminants such as the nonplanar PCBs. This study also sheds light on the heretofore puzzling finding that many classes of antihormones, in addition to antiglucocorticoids, induce CYP3A (Kocarek *et al.*, 1995) by demonstrating that these steroids activate PXR. Given the high concentrations of steroids and xenobiotics required to activate PXR and the structural and pharmacological diversity among the activators, it is likely that PXR-mediated steroid and xenobiotic activation of CYP3A is primarily for the purpose of metabolizing these foreign molecules.

Both the organochlorine pesticides and the polychlorinated biphenyls are ubiquitous environmental contaminants. We demonstrated previously that nonplanar PCB congeners induced *de novo* synthesis of CYP3A and induced CYP2B in primary rat hepatocyte cultures and in rat liver *in vivo* (Schuetz *et al.*, 1986). Our data herein provide the mechanistic connection demonstrating that these same PCBs use PXR to transcriptionally activate the *CYP3A23* DR-3 and, in general, parallel our previous findings. Indeed, it is possible

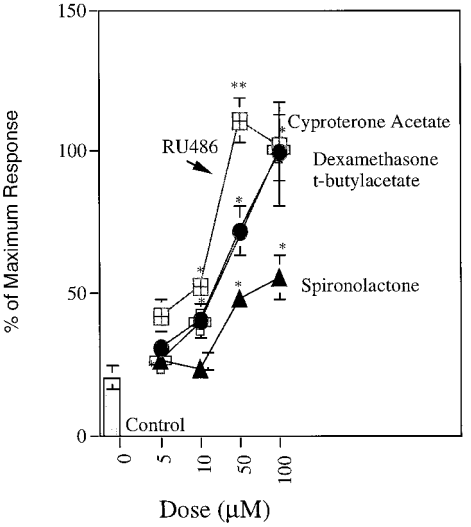

Fig. 3. Steroid antihormones activate PXR. LLC-PK1 cells co-transfected as in Fig. 1 were treated with various concentrations of dexamethasone-t-butylacetate (⊕) or spironolactone (▲), or RU486 (⊞), or cyproterone acetate (●), or not treated with steroid (*control*). Cell extracts were assayed for CAT and Luciferase activities. Data was graphed as the percentage of maximal response (100 μM dexamethasone-*t*-butylacetate) and values represent means ± standard deviation from two to four independent experiments with three to four replicates per treatment per experiment. The student's *t* test was used to determine whether the treated values were significantly different from the control, with $p < 0.05$ as the limit of significance. *, $p < 0.05$; **, $p < 0.005$.

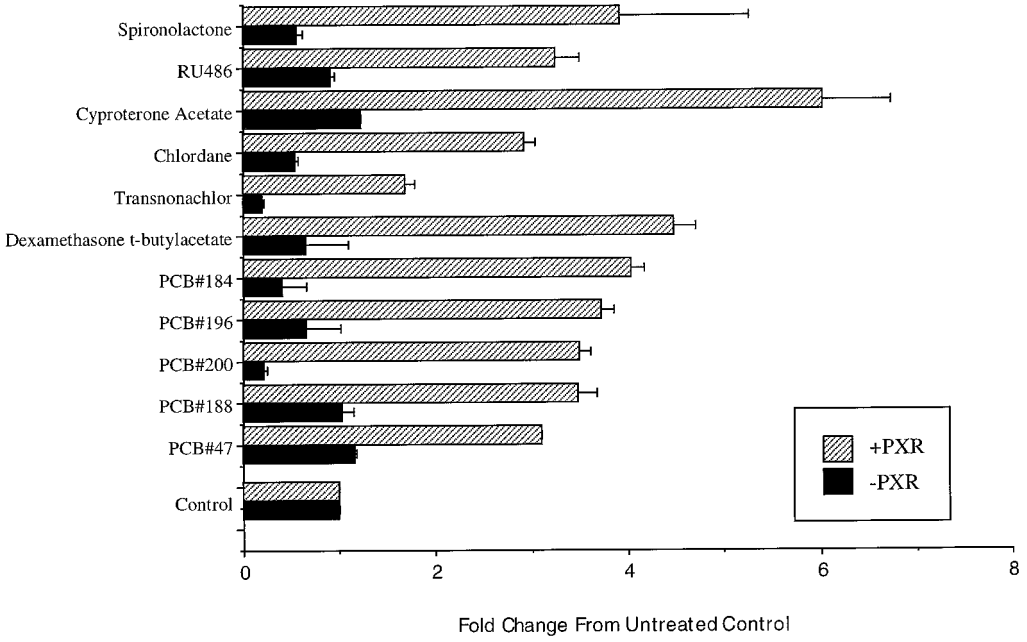

Fig. 4. PXR is required for xenobiotic and steroid induction of *CYP3A23*. 200,000 LLC-PK1 cells were transfected with $(CYP3A23)_2$-tk-CAT and TK-LUC with or without co-transfected PXR and then treated 24 hr with steroids (100 μM) except dexamethasone *t*-butylacetate (50 μM), organochlorine pesticides (20 μM), or PCBs (50 μM). Data is graphed as -fold change from untreated control 1.00 ± 0.009 (variation between measurements was within the size of the symbol) determined as in Fig. 1. Values represent means ± standard deviation from three to four replicates per treatment per experiment (representative of two to three independent experiments).

that, in addition to activation of CYP3A, some biological activities of the nonplanar congeners, including toxicities, are mediated by interactions of these PCBs with PXR. PCBs cause liver hypertrophy, are tumor promoters, and cause neurotoxicity and disruptions in calcium regulation (Hansen, 1998). PXR-mediated PCB activation of the *CYP3A23* DR-3 may be a predictive biomarker correlated with some PCB toxicities. Thus, dose-response analysis of PCB congeners or mixtures of PCB for PXR-mediated activation of the *CYP3A23* DR-3 may provide an assay to rapidly segregate toxic and nontoxic nonplanar PCBs. Interestingly, the antiandrogen cyproterone acetate, identified herein as a PXR activator, also includes liver cell proliferation, and previous attempts to determine the signaling pathway for proliferation have been futile (Menegazzi *et al.*, 1997). Cyproterone acetate also causes DNA damage in liver (Werner *et al.*, 1997), is a rodent liver tumor promoter (Duivenvoorden *et al.*, 1995), and increases sensitivity of hepatocytes to undergo apoptosis (Oberhammer *et al.*, 1996). It remains to be determined whether ligand activated PXR is involved in mediating any of these other biological activities associated with nonplanar PCBs or cyproterone acetate.

We conclude that PXR mediates induction of the *CYP3A23* DR-3 by antihormones and environmental xenobiotics. Thus, PXR joins the aryl hydrocarbon receptor in being a transcription factor ligand-activated by multiple classes of agents (Nebert, 1989; Dzeletovic *et al.*, 1997). By analogy to the aryl hydrocarbon receptor paradigm, it will be of interest in the future to determine whether individual differences in PXR ligand affinity among humans correlates with CYP3A inducibility and, like P-glycoprotein (Schuetz *et al.*, 1996), also contributes to the wide variation in the CYP3A inductive response (Watkins *et al.*, 1989; Kolars *et al.*, 1992).

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