

Environmental phthalate monoesters activate pregnane X receptor-mediated transcription

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Abstract

Phthalate esters, widely used as plasticizers in the manufacture of products made of polyvinyl chloride, induce reproductive and developmental toxicities in rodents. The mechanism that underlies these effects of phthalate exposure, including the potential role of members of the nuclear receptor superfamily, is not known. The present study investigates the effects of phthalates on the pregnane X receptor (PXR), which mediates the induction of enzymes involved in steroid metabolism and xenobiotic detoxification. The ability of phthalate monoesters to activate PXR-mediated transcription was assayed in a HepG2 cell reporter assay following transfection with mouse PXR (mPXR), human PXR (hPXR), or the hPXR allelic variants V140M, D163G, and A370T. Mono-2-ethylhexyl phthalate (MEHP) increased the transcriptional activity of both mPXR and hPXR (5- and 15-fold, respectively) with EC_{50} values of 7–8 μ M. mPXR and hPXR were also activated by monobenzyl phthalate (MBzP, up to 5- to 6-fold) but were unresponsive to monomethyl phthalate and mono-*n*-butyl phthalate (M(n)BP) at the highest concentrations tested (300 μ M). hPXR-V140M and hPXR-A370T exhibited patterns of phthalate responses similar to the wild-type receptor. By contrast, hPXR-D163G was unresponsive to all phthalate monoesters tested. Further studies revealed that hPXR-D163G did respond to rifampicin, but required approximately 40-fold higher concentrations than wild-type receptor, suggesting that the ligand-binding domain D163G variant has impaired ligand-binding activity. The responsiveness of PXR to activation by phthalate monoesters demonstrated here suggests that these ubiquitous environmental chemicals may, in part, exhibit their endocrine disruptor activities by altering PXR-regulated steroid hormone metabolism with potential adverse health effects in exposed individuals.

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Keywords: PXR; MEHP; Phthalate monoesters

Introduction

Phthalate esters are widely used as plasticizers in the manufacture of products made of flexible polyvinyl chloride products, including medical bags and food packaging, and can also be found in a variety of industrial fixatives, detergents, cosmetics, and solvents (Blass,

1992). Phthalates are ubiquitous environmental contaminants with a potential for human exposure by oral, dermal, inhalation, and intravenous routes (Huber et al., 1996). The commonly used plasticizer di-(2-ethylhexyl) phthalate (DEHP) is a rodent reproductive toxicant as well as a teratogen and liver carcinogen (Doull et al., 1999). Mono-2-ethylhexyl phthalate (MEHP), the mono-ester hydrolysis product of DEHP, is a hepatic peroxisome proliferator chemical whose peroxisome proliferative and hepatocarcinogenic effects are mediated by the nuclear receptor PPAR α (Ward et al., 1998). In vitro studies demonstrate that MEHP can activate PPAR α (Maloney and Waxman, 1999) and can induce the expression of several endogenous PPAR α target genes in a liver-derived rodent cell line (Hurst and Waxman, 2003). However, the renal and testicular toxicities induced by DEHP exposure are largely PPAR α -independent (Ward et

Abbreviations: DEHP, di-2-ethylhexyl phthalate; DMEM, Dulbecco's Modified Eagle Medium; DMSO, dimethyl sulfoxide; DR3, direct repeat with 3 nucleotide spacer; EC_{50} , effective concentration for half-maximal response; ER6, everted repeat with 6 nucleotide spacer; FBS, fetal bovine system; MBzP, monobenzyl phthalate; MBuP, mono-*sec*-butyl phthalate; MEHP, mono-2-ethylhexyl phthalate; M(n)BuP, mono-*n*-butyl phthalate; Pregnenolone, 5-pregnen-3 β -ol-20-one; PXR, pregnane X receptor.

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al., 1998), which raises the possibility that these and other toxicological effects of phthalate esters may be mediated by other nuclear receptors, including other PPAR isoforms such as PPAR γ (Hurst and Waxman, 2003; Maloney and Waxman, 1999) and PPAR δ (Lampen et al., 2003).

Another candidate receptor for phthalates is the nuclear receptor superfamily member pregnane X receptor (PXR), which is activated by structurally diverse xenobiotics including various pharmaceutical compounds, carcinogens, and environmental contaminants such as organochloride pesticides, polychlorinated biphenyls, and the anti-androgen cyproterone acetate (Schuetz et al., 1998). PXR can also be activated by the endocrine disrupting chemicals nonylphenol and phthalic acid (Masuyama et al., 2000). PXR regulates a broad range of genes involved in the detoxification and elimination of structurally diverse drugs and environmental chemicals (Goodwin et al., 2002; Waxman, 1999). PXR also regulates the metabolism of toxic bile acids by inducing the expression of CYP3A enzymes involved in the detoxification of lithocholic acid (Xie et al., 2001). Steroid hormone metabolism can also be regulated by PXR (Goodwin et al., 2002), for example, by increasing the expression of the phase II conjugation enzyme UDP-glucouronosyltransferase 1A, which contributes to estrogen metabolism (Xie et al., 2003). Conceivably, activation of PXR by phthalate monoesters may disrupt normal hormone signaling pathways during critical periods of development leading to developmental and reproductive toxicities associated with phthalate exposure.

Presently, we use a HepG2 cell-based reporter-gene assay to investigate the responsiveness of PXR to MEHP and other environmental phthalate monoesters with significant human exposure (Blount et al., 2000). We compare the responsiveness of mouse PXR (mPXR) and human PXR (hPXR) which exhibit important species-specific differences in ligand specificity as a result of sequence divergence in the COOH-terminal ligand-binding domain (Moore et al., 2002). We also evaluate the phthalate responsiveness of several naturally occurring allelic variants of hPXR (Hustert et al., 2001) to gain insight into the possible genetic mechanisms that may contribute to interindividual differences in human responsiveness to this class of environmental chemicals.

Materials and methods

Chemicals. Monobenzyl phthalate (MBzP), mono-*sec*-butyl phthalate (MBuP), monomethyl phthalate, and mono-*n*-butyl phthalate (M(n)BP) were purchased from Aldrich Chemical Co. (Milwaukee, WI). MEHP was obtained from TCI America (Portland, OR). Phthalic acid, rifampicin, and pregnenolone (5-pregnen-3 β -ol-20-one) were obtained from Sigma (St. Louis, MO).

Plasmids. The mouse PXR expression plasmid pSG5-mPXR₁ and human PXR expression plasmid pSG5-hPXR were obtained from Dr. Steven Kliewer (Glaxo-Smith-Kline, Research Triangle Park, NC). The PXR-activated firefly

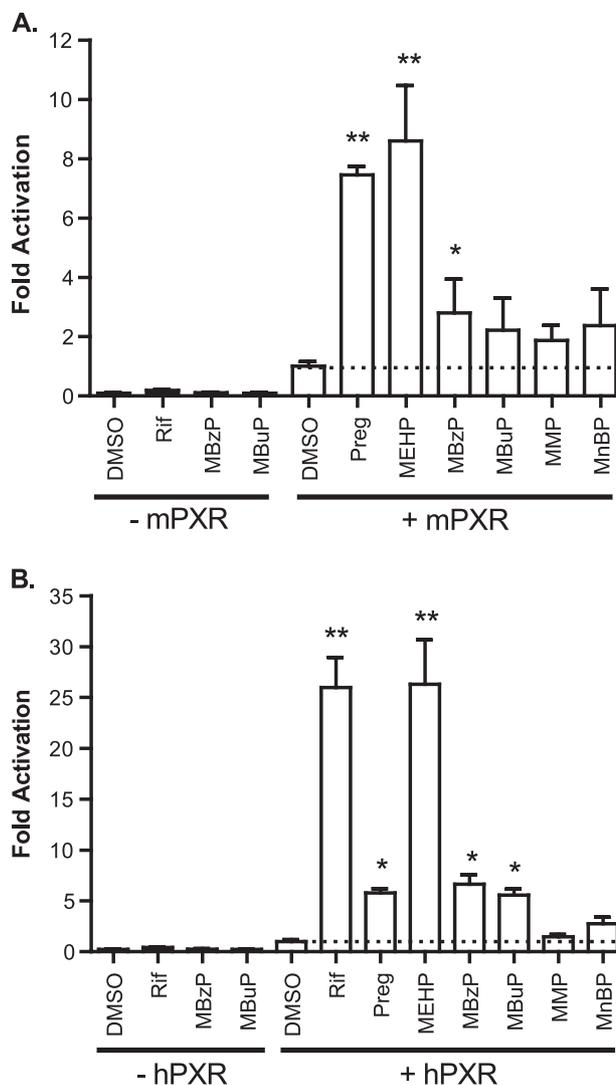


Fig. 1. Activation of mPXR and hPXR in response to phthalate monoester treatment. HepG2 cells were transfected with expression plasmid encoding mPXR (panel A), hPXR (panel B), or no PXR plasmid, as indicated. All cells were co-transfected with a CYP3A4-Luc reporter plasmid (pGL3-CYP3A4(-7830Δ7208/-364)) and a *Renilla* luciferase internal control plasmid (pRL-CMV). Treatment of cells for 24 h with the indicated chemicals and determination of firefly luciferase activity normalized to the *Renilla* luciferase internal control were carried out as described in Materials and methods. Concentrations used were Rif, rifampicin (10 μ M); MBzP (300 μ M); MBuP (300 μ M); Preg, pregnenolone (25 μ M); MEHP (50 μ M); MMP, monomethyl phthalate (300 μ M); and 300 μ M M(n)BP. Data shown are luciferase reporter values normalized to PXR-transfected cells treated with 0.1% DMSO (vehicle) as a control (horizontal dashed line). Normalized CYP3A4-luciferase activity was approximately ~2-fold higher in mPXR transfected, DMSO-treated cells than in similarly treated hPXR transfected cells (data not shown), and consequently, fold-activation values were routinely higher in the case of hPXR. Data shown are mean \pm SD values, $n = 3$ based on a single representative experiment. * $P < 0.05$; ** $P < 0.01$ from DMSO control values by ANOVA.

luciferase reporter genes (3A4)₃-tk-luc, which contains three copies of an ER6 response element derived from the human CYP3A4 gene, and (3A23)₃-tk-luc, which contains three copies of a DR3 response element derived from the rat CYP3A23 gene, were obtained from Dr. Ron Evans (Salk Institute, La Jolla, CA) (Xie et al., 2000). Expression plasmids for wild-type hPXR (pcDNA3-hPXR) and three of its naturally occurring allelic variants (hPXR-V140M, hPXR-D163G, hPXR-A370T) were obtained from Dr. Oliver Burk (Fischer-Bosch Institute of Clinical Pharmacology, Stuttgart, Germany) (Hustert et al., 2001). The PXR-activated CYP3A4 promoter-derived firefly luciferase reporter plasmid pGL3-CYP3A4(-7830Δ7208/-364) ('CYP3A4-luciferase'), which contains two naturally occurring ER6 elements and one DR3 element derived from the CYP3A4 promoter (see Fig. 4C, below), was also obtained from Dr. Burk. The *Renilla* luciferase reporter plasmid pRL-CMV was purchased from Promega (Madison, WI).

Cell culture and transient transfections. HepG2 cells (ATCC, Manassas, VA) were grown in 75 cm² tissue culture flasks in Dulbecco's Modified Eagle Medium (DMEM) supplemented with 10% fetal bovine serum (FBS, Sigma) and 50 U/ml penicillin–streptomycin (Invitrogen, Carlsbad, CA). Cells were cultured overnight at 37 °C and then trypsinized and reseeded at 75 000 cells/well in a 48-well

plate (Corning Inc., Corning, NY) in DMEM containing 10% FBS. Twenty-four hours later, the cells were transfected with a PXR expression plasmid (5 ng/well), a PXR reporter plasmid (90 ng/well), and pRL-CMV (0.5 ng/well) for normalization of transfection efficiency, using FuGENE 6 transfection reagent (Boehringer-Mannheim, Germany) and methods described previously (Hurst and Waxman, 2003). Cells were then stimulated for 24 h with rifampicin (10 μM), pregnenolone (25 μM), or phthalate esters dissolved in dimethyl sulfoxide (DMSO) at concentrations indicated in each figure (final DMSO concentration = 0.1%). Following phthalate treatment for 24 h, cells were lysed by incubation at 4 °C in 200 μl passive lysis buffer (Promega) for 20 min. Firefly and *Renilla* luciferase activities were measured in the cell lysate using a dual reporter assay system (Promega) and a Monolight 2010 luminometer (Analytical Luminescence Laboratory, San Diego, CA). Data shown in each figure are presented as mean ± SD (*n* = 3 replicates). Each figure is representative of at least 2–3 independent replicate experiments. EC₅₀ values were calculated using GraphPad Prism software, version 3.0 (GraphPad, San Diego, CA). Data shown for hPXR expression are based on transfection experiments using the plasmid pcDNA3-hPXR. Comparable results in terms of fold-activation in response to rifampicin and phthalate monoesters were obtained using pSG5-hPXR. In transfec-

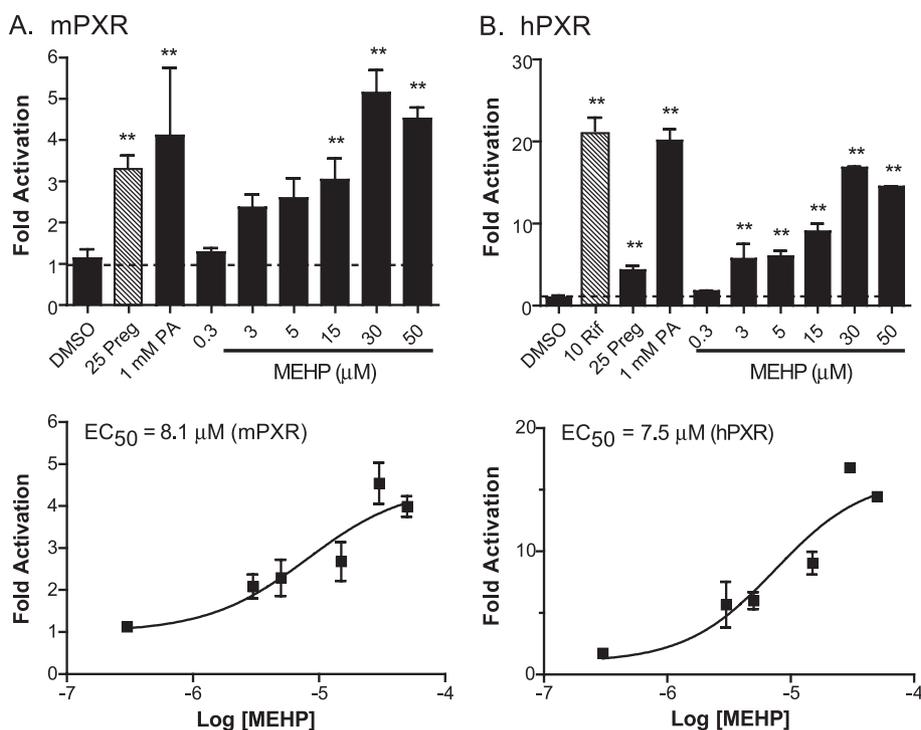


Fig. 2. Dose-response for activation of PXR by MEHP. HepG2 cell transfection with mPXR (panel A) or hPXR (panel B), CYP3A4-luciferase reporter, and pRL-CMV, followed by treatment of cells with DMSO (vehicle) (horizontal dashed line) or the indicated PXR agonists, and determination of relative luciferase activity values were carried out as described in Fig. 1. Data shown are fold activation values based on normalized luciferase reporter activities. PA, phthalic acid. ***P* < 0.01 from DMSO control values by ANOVA. EC₅₀ values were calculated using non-linear regression analysis (GraphPad Prism v3.0) based on *n* = 3–6 independent samples per MEHP concentration (lower set of panels).

tion experiments not shown, the use of DMEM containing 10% charcoal-stripped, delipidated FBS (Sigma) decreased basal CYP3A4-luciferase activity approximately 2-fold compared to experiments in which unmodified FBS was used. However, no change in the relative ability of phthalate monoesters to activate hPXR was observed.

Statistical analysis. GraphPad Prism, v3.0 was used to perform all statistical analyses. A one-way analysis of variance (ANOVA) followed by Dunnett's post hoc test was used to determine whether differences between phthalate treatments were significantly different from control values, with $P < 0.05$ as the limit of significance.

Results

Effect of phthalate monoesters on PXR activity

The *trans*-activation of PXR by phthalate monoesters was investigated in HepG2 cells transfected with mouse or human PXR expression plasmid and a PXR-responsive CYP3A4-luciferase reporter. Cells were treated for 24 h with phthalates previously shown to activate PPAR α and PPAR γ (Hurst and Waxman, 2003; Maloney and Waxman, 1999). Pregnenolone (a naturally occurring steroid) and rifampicin (an antibiotic) were used as positive controls for the activation of mPXR and hPXR, respectively (Figs. 1A and B). Rifampicin (10 μ M) was a highly efficacious activator of hPXR (≥ 20 -fold increase in reporter activity vs. control cells treated with DMSO). mPXR and hPXR were both activated approximately 6- to 7-fold by pregnenolone (25 μ M) (Figs. 1A and B). Basal CYP3A4-luciferase reporter activity in PXR-transfected cells treated with DMSO vehicle was 10-fold higher than in DMSO-treated untransfected cells, indicating the presence of endogenous PXR activator(s) in cells or culture medium (Figs. 1A and B). A 2- to 3-fold increase in CYP3A4-luciferase reporter activity was seen with rifampicin in the absence of transfected PXR, suggesting the presence of a low level of endogenous PXR (Fig. 1A, bar 2 vs. bar 1).

We next examined several phthalate monoesters for their ability to activate mouse and human PXR. Treatment of the PXR-transfected cells with MEHP led to a substantial increase in PXR-dependent reporter activity relative to DMSO (control)-treated cultures. mPXR (Fig. 1A) and hPXR (Fig. 1B) were both activated by MEHP to a level similar to that obtained following treatment of cells with pregnenolone (mPXR) and rifampicin (hPXR), respectively. MBzP and MBuP increased mPXR transcriptional activity up to approximately 2- to 3-fold (Fig. 1A), while hPXR activity was activated 5- to 6-fold by these phthalates (Fig. 1B). Overall, the fold-activation values were lower with mPXR than with hPXR, in a large part reflecting the approximately 2-fold higher basal luciferase activity seen with mPXR in DMSO-treated cells. No significant *trans*-

activation of mouse or human PXR was detected following treatment with monomethyl phthalate or M(n)BP. In control samples in the absence of transfected mPXR or hPXR, no activation of the CYP3A4-luciferase reporter was observed with phthalate monoester treatment, demonstrating that the transcriptional responses seen in these experiments are PXR-dependent (Fig. 1, bar 3 and bar 4).

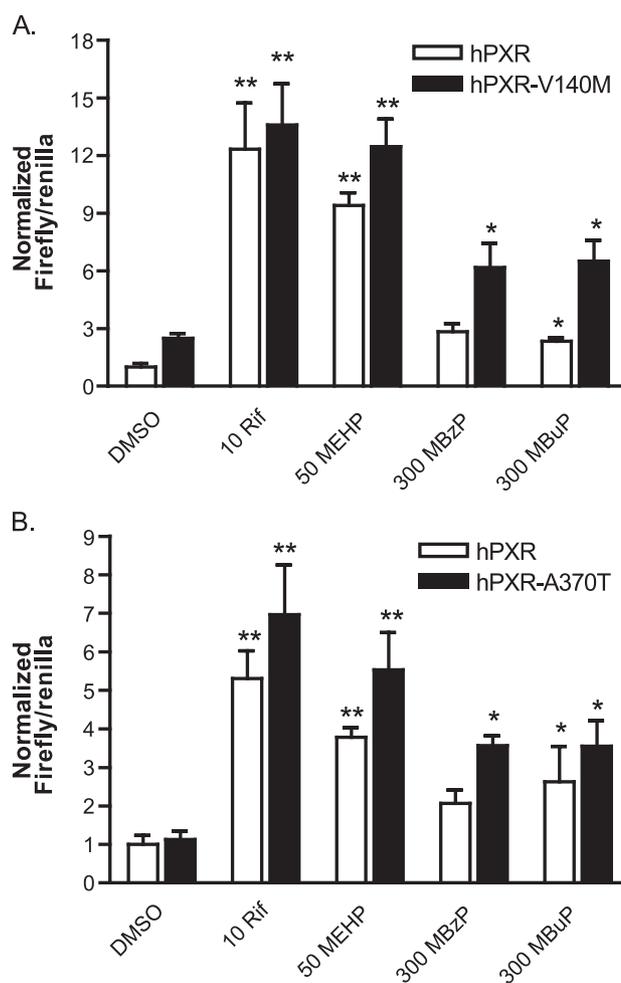


Fig. 3. Phthalate monoester responsiveness of hPXR allelic variants. HepG2 cells were co-transfected with CYP3A4-luciferase reporter plasmid, pRL-CMV internal control plasmid, and wild-type hPXR or hPXR variant expression plasmids, as indicated. Treatment of cells with the indicated concentrations (μ M) of phthalates for 24 h and determination of firefly luciferase activity normalized to the *Renilla* luciferase internal control were carried out as in Fig. 1. The firefly/*Renilla* luciferase ratio was set to 1 for DMSO-treated wild-type hPXR. hPXR-V140M showed an approximately 2.5-fold higher firefly/*Renilla* luciferase ratio (panel A), while hPXR-A370T showed a ratio undistinguishable from wild-type hPXR. Thus, with rifampicin, both hPXR variants showed absolute induced activities similar to wild-type hPXR. Although the absolute firefly/*Renilla* luciferase values for MBzP and MBuP were somewhat higher for hPXR-V140M than with wild-type hPXR, the fold activation was similar to that of hPXR due to higher basal activity with the hPXR-V140M variant. Rif, rifampicin. * $P < 0.05$; ** $P < 0.01$ from DMSO control values by ANOVA. MB₂P activation of hPXR was statistically significant in the experiment shown in Fig. 1B but not in this experiment.

Dose-response studies revealed EC_{50} values of 7–8 μ M for both mPXR and hPXR (Fig. 2). Maximal *trans*-activation of mPXR (4- to 5-fold) was less robust than that of hPXR (12- to 15-fold), reflecting the higher basal activity of mPXR, as noted above. Both receptors were also activated by phthalic acid, which induced a 4-fold increase in mPXR activity and a 20-fold increase in hPXR activity (Fig. 2, PA; top panels). *Trans*-activation by phthalic acid was characterized by EC_{50} values of 0.7 and 3 μ M for mPXR and hPXR, respectively (data not shown).

Impact of PXR allelic variation on phthalate-induced receptor *trans*-activation

Several naturally occurring hPXR allelic variants have been characterized, one of which exhibits an altered response to certain PXR ligands (Hustert et al., 2001). We therefore investigated whether these human PXR protein variants exhibit altered activity in response to phthalate monoester treatment. Two of the hPXR variants tested, hPXR-V140M and hPXR-A370T, contain single amino acid substitutions near or within the receptor's ligand-binding domain. hPXR-V140M responded to both rifampicin and MEHP in a manner similar to wild-type hPXR (Fig. 3A); however, the fold-activation values were lower for the V140M variant because of its higher basal CYP3A4-luciferase activity (Fig. 3A, compare first two bars). Maximal activation of hPXR-A370T by rifampicin and MEHP was similar to that of hPXR (Fig. 3B). MBzP and MBuP activated hPXR and its V140M and A370T variants 2- to 4-fold (Figs. 3A and B).

We next investigated the activation of a third hPXR variant, hPXR-D163G, by phthalate monoesters. Previous studies demonstrated that the *trans*-activation profile of this PXR variant is influenced by the organization of the PXR response element (Hustert et al., 2001), which can be either a direct repeat 3 (DR3) or an everted repeat 6 (ER6) based on the consensus sequence AG(G/T)TCA (Mangelsdorf et al., 1995). We therefore characterized the *trans*-activation of hPXR-D163G in comparison to wild-type hPXR using three different PXR reporters: tk(3A23)₃-luciferase, which contains three copies of a DR3 PXR response element, tk(3A4)₃-luciferase, which contains three copies of an

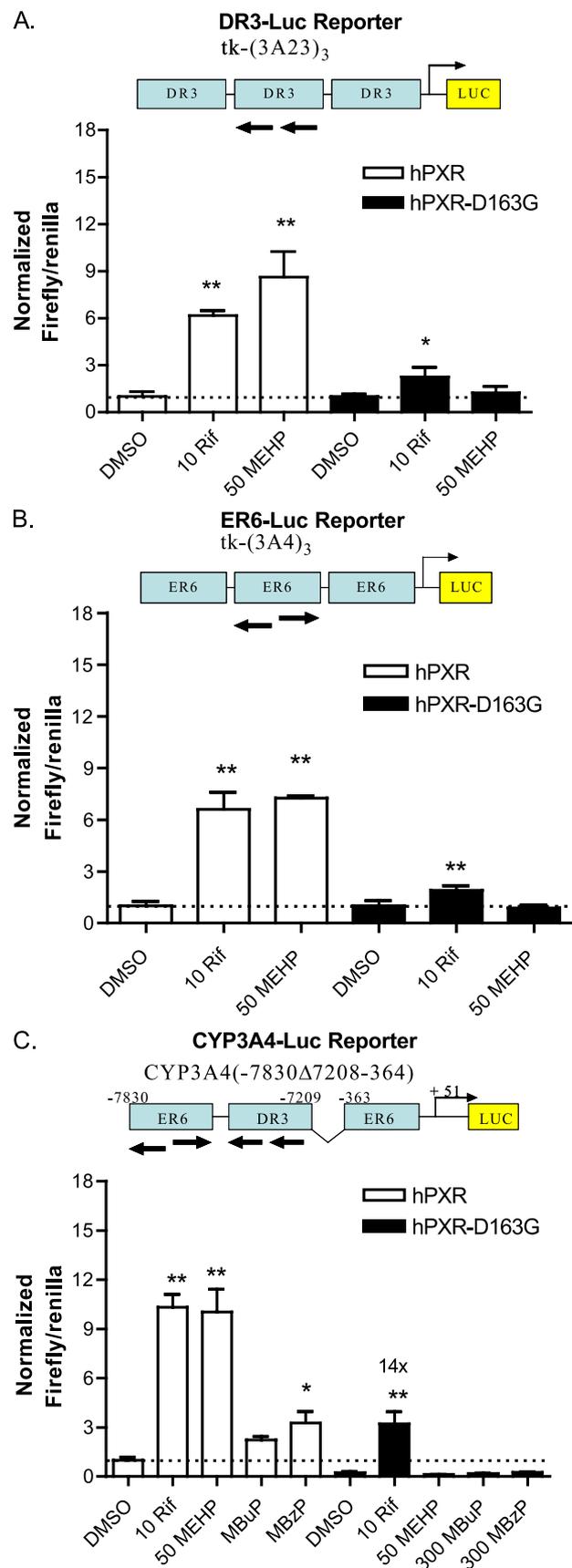


Fig. 4. Activation of hPXR-D163G assayed using different PXR reporters. HepG2 cells were co-transfected with hPXR or hPXR-D163G expression plasmid, pRL-CMV, and the indicated PXR reporter plasmids. Cells were stimulated with the indicated micromolar concentrations of rifampicin (Rif), MEHP, MBzP, or MBuP. Data shown are luciferase reporter values normalized to untreated DMSO controls (horizontal dashed line), mean \pm SD for $n = 3$ independent samples from a single representative experiment. No difference in basal activities (i.e., in DMSO-treated cells) was observed with the DR3 and ER6 reporters in wild-type hPXR-transfected cells compared to hPXR-D163G-transfected cells (panels A and B). Normalized CYP3A4-luciferase basal activity measured in hPXR-D163G-transfected cells was approximately 5-fold lower than that of hPXR-transfected cells (panel C). * $P < 0.05$; ** $P < 0.01$ from DMSO control values by ANOVA.

ER6 element, and CYP3A4(-7830Δ7208/-364)-luciferase, which was used in all of the experiments presented above and contains one DR3 and two ER6 elements in their natural CYP3A4 promoter context (Fig. 4). Co-transfection experiments using the DR3-luciferase reporter demonstrated that hPXR-D163G was substantially less responsive than hPXR to treatment with rifampicin and was totally unresponsive to MEHP (Fig. 4A). hPXR-D163G was also substantially less active than wild-type hPXR with the ER6 reporter (Fig. 4B). In cells co-transfected with hPXR-D163G and the CYP3A4-luciferase reporter, decreased activity was observed in rifampicin-stimulated cells compared to wild-type hPXR; however, the fold-activation (14-fold) was substantially higher due to a decrease in basal activity of the hPXR-D163G receptor (i.e., in the absence of exogenous ligand) compared to wild-type PXR. MEHP, MBzP, and MBuP were unable to activate hPXR-D163G, even when assayed using the CYP3A4-luciferase reporter.

Decreased sensitivity of hPXR-D163G to activation by rifampicin

The decreased basal activity and increased fold-activation of hPXR-D163G following rifampicin treatment seen in Fig. 4C and reported earlier using the same CYP3A4-luciferase reporter (Hustert et al., 2001) suggest that the binding of endogenous PXR activators, as well as exogenous PXR ligands, may be altered with this receptor variant. To test this possibility, we characterized the dose response for rifampicin activation of hPXR-D163G. Maximal *trans*-activation of hPXR-D163G (≥ 75 -fold activation at 50 μM rifampicin) was substantially higher than that of hPXR (10- to 12-fold activation), largely reflecting the lower basal activity of the D163G variant (Figs. 5A and B). However, hPXR-D163G was much less sensitive to rifampicin stimulation than wild-type hPXR, requiring 40-fold higher rifampicin concentrations ($EC_{50} = 20 \mu\text{M}$) compared to

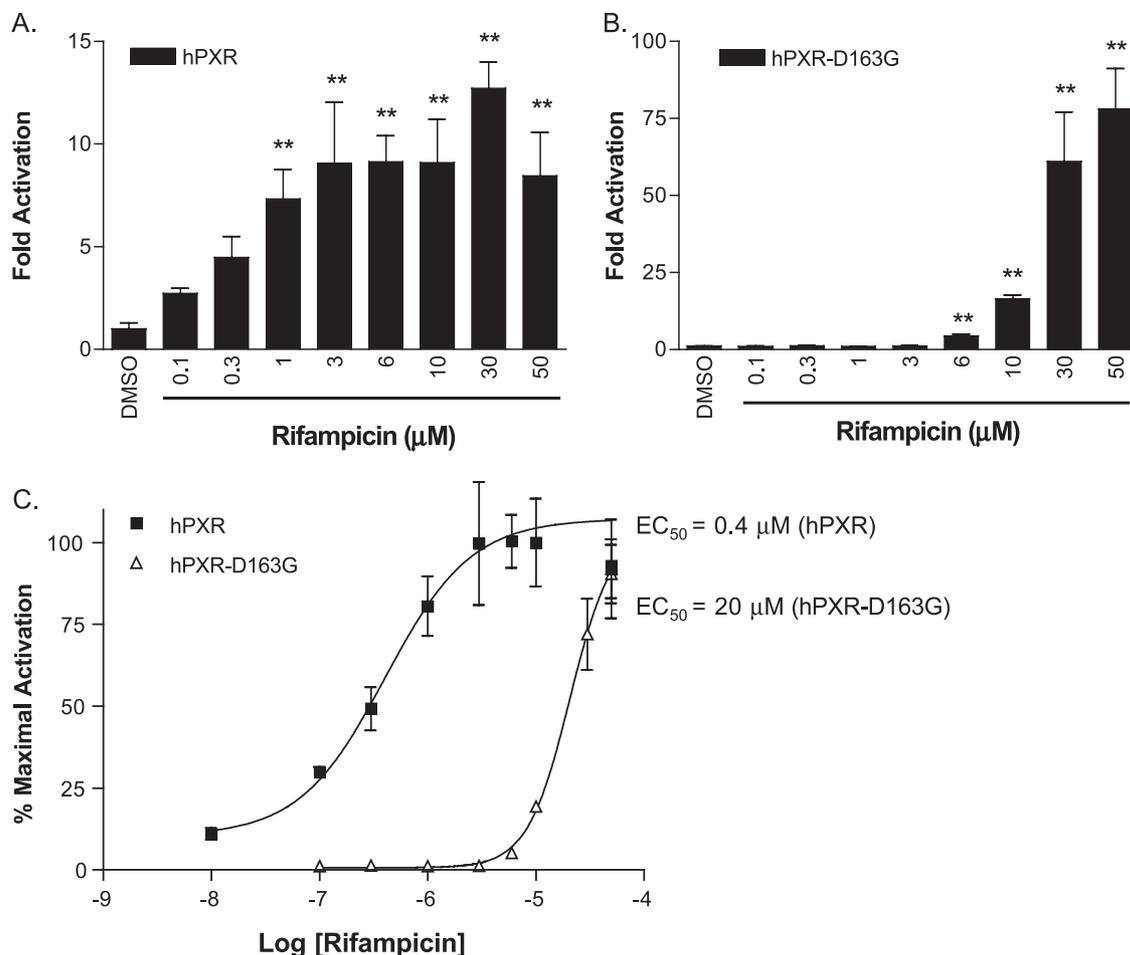


Fig. 5. Dose response for activation of PXR by rifampicin. HepG2 cell transfection using hPXR (panel A) or hPXR-D163G (panel B) and CYP3A4-luciferase reporter, treatment with rifampicin at the indicated concentrations, and determination of relative luciferase values were carried out as described in Fig. 4C. Data shown are based on normalized luciferase reporter activities relative to DMSO controls, mean \pm SD, $n = 3$ based on a single representative experiment. Rifampicin (50 μM) was an efficacious activator of hPXR-D163G (≥ 75 -fold increase in activity) (panel B). Lower concentrations of rifampicin (10 μM) increased transcriptional activity of hPXR-D163G (approximately 14-fold) (Fig. 4C). Maximal activation for each receptor (hPXR, panel A; hPXR-D163G, panel B) was arbitrarily set as 100%. EC_{50} values (panel C) were calculated using non-linear regression analysis (GraphPad Prism v3.0). $*P < 0.05$; $**P < 0.01$ from DMSO control values by ANOVA.

hPXR ($EC_{50} = 0.4 \mu\text{M}$) to achieve half-maximal reporter activity (Fig. 5C). Thus, the hPXR-D163G variant exhibits impaired activity with phthalate monoesters, rifampicin, and endogenous cellular PXR activators.

Discussion

PXR acts as a xenobiotic sensor that regulates a broad range of genes involved in the transport, metabolism, and elimination of steroids, bile acids, and foreign chemicals. In this report, we describe the responsiveness of PXR to MEHP and other environmental phthalates with significant human exposure. Based on their *trans*-activation profiles in cell-based reporter assays, several phthalate monoesters, including MEHP and MBzP, were shown to stimulate mPXR and hPXR transcriptional activity. Furthermore, these phthalate monoesters activated two naturally occurring allelic variants of hPXR. However, a third variant, hPXR-D163G, was unresponsive to phthalate treatment.

Unlike classic members of the steroid-nuclear receptor superfamily, which are typically responsive to one or a limited number of naturally occurring, high-affinity ligands (K_d approximately in the nanomolar range), PXR is a promiscuous nuclear receptor that responds to structurally diverse ligands, including many foreign chemicals, with dissociation constant values in the micromolar range (Jones et al., 2000). In the present study, mPXR and hPXR were both activated by the widespread environmental phthalate MEHP with EC_{50} values of 7–8 μM . These values are within the range of peak plasma MEHP concentrations that induce reproductive and developmental toxicities in rodents (Pollack et al., 1985), indicating that PXR is activated by MEHP at concentrations that induce toxicity. However, rodent toxicities associated with phthalate exposure are unlikely to be due to activation of PXR alone, rather, they may result from the activation of multiple nuclear receptors leading to changes in steroid hormone metabolism. PXR exhibits tissue-specific expression in both rodents and humans, and is most highly expressed in liver, with lesser amounts present in the colon and small intestine (Goodwin et al., 2002). Previous studies demonstrated a significant increase in the level of PXR mRNA in the liver and ovary of pregnant mice between gestation day 12 and 19, suggesting that PXR may act as a steroid sensor that protects the fetal–maternal system from endogenous steroids and toxic xenobiotics (Masuyama et al., 2001). Although PPAR α , and not PXR, mediates the liver toxicity of MEHP in rodents (Ward et al., 1998), the activation of PXR by phthalates during critical periods of development may lead to subtle changes in steroid hormone metabolism that impact on sensitive tissues and induce the widely observed gonadal and reproductive defects associated with this class of chemicals.

In addition to the activation of PXR described here, MEHP and other phthalate monoesters can activate PPARs α , γ , and δ (Hurst and Waxman, 2003; Lampen et al., 2003;

Maloney and Waxman, 1999), potentially leading to effects on steroidogenesis. MEHP is a primary metabolite of DEHP; it inhibits granulosa cell estradiol production in preovulatory follicles by a mechanism that involves suppression of aromatase mRNA, protein, and enzyme activity (Davis et al., 1994) in a manner dependent on PPAR α and PPAR γ (Lovekamp-Swan et al., 2003). Moreover, butylbenzyl phthalate, the parent compound of MBzP, possesses estrogenic properties (Picard et al., 2001; Zacharewski et al., 1998), although in the reports cited, none of the butylbenzyl phthalate metabolites, including mono-*n*-butyl phthalate, monobenzyl phthalate, and phthalic acid, displayed estrogenic effects when tested in vitro (Picard et al., 2001). Furthermore, butylbenzyl phthalate stimulates transcriptional activity of estrogen receptor- α , but not estrogen receptor- β (Fujita et al., 2003). Taken together, these findings suggest that the toxicities associated with phthalate exposure may involve multiple nuclear receptors acting within several tissues.

PXR regulates members of the cytochrome P450 3A (CYP3A) superfamily, which are most highly expressed in the liver and gut, and are induced by a diverse spectrum of foreign chemicals. CYP3A4 is the most abundant P450 form in human liver, but its expression levels can vary approximately 60-fold among individuals (Shimada et al., 1994). PXR also regulates the expression of other genes involved in xenobiotic metabolism, including CYP2C8, CYP2C9, CYP2B6 (Honkakoski and Negishi, 2000), organic anion transporter protein 2 (Staudinger et al., 2001), P-glycoprotein–multidrug resistance protein 1 (Geick et al., 2001), and several genes important for bile acid metabolism (Watkins et al., 2002). Collectively, these data support a role of PXR in hepatoprotection in response to foreign chemicals. Given the wide variations in PXR responses observed in human populations (Guengerich, 1999), exposure to phthalate monoesters or other PXR-activating endocrine disruptor chemicals may contribute to the observed interindividual variability in the expression of CYP3A and other PXR target genes.

The differential sensitivity of individual humans to MEHP (NTP, 2000) could, in part, reflect genetic polymorphisms in PXR. In the present study, we evaluated the responsiveness of several naturally occurring allelic variants of PXR (Hustert et al., 2001) to help elucidate interindividual differences in human PXR responses to phthalates. Three of the known PXR variants (V140M, D163G, and A370T) incorporate coding region changes within or near the ligand-binding domain of hPXR and exhibit altered *trans*-activation of PXR reporter genes. The allelic frequency of these variants is low, however, ranging from 0.2% to 1.5%, with hPXR-V140M found in Caucasians and the D163G and A370T variants present in Africans (Hustert et al., 2001). hPXR-V140M and hPXR-A370T were fully responsive to rifampicin and MEHP when compared to wild-type hPXR. By contrast, hPXR-D163G was totally unresponsive to all of the phthalates tested. hPXR-D163G

also displayed approximately 40-fold lower intrinsic sensitivity to activation by rifampicin, suggesting that its ligand-binding domain exhibits impaired binding of rifampicin as well as phthalates. Although the rarity of the hPXR-D163G polymorphism (1.4% allelic frequency in Africans; Hustert et al., 2001) suggests that this mutation alone cannot account for the observed interindividual variability in CYP3A4 expression or activity, other heretofore unidentified PXR variants may significantly influence CYP3A4 activity and alter xenobiotic metabolism leading to adverse consequences. hPXR-D163G displayed higher fold-activation in rifampicin-stimulated cells compared to wild-type receptor, and this apparent increase in activity could be fully explained by an approximately 5-fold lower basal activity of the hPXR-D163 receptor in the absence of exogenous PXR activators. These findings are consistent with those of Hustert et al. (2001), who reported that the increased activity of hPXR-D163G was dependent on the ER6 element, two copies of which are present in the CYP3A4-luciferase reporter. In our experiments, however, a reporter containing three copies of an isolated CYP3A4 promoter ER6 element did not display enhanced activity with rifampicin-activated hPXR-D163G. Further work is needed to fully elucidate the molecular basis for the altered activity exhibited by hPXR-D163G and the promoter element-dependence of its activity.

There is great interest in understanding the human health impact of environmental chemicals that may alter steroid metabolism or the detoxification and elimination of xenobiotics by interfering with the regulation of genes by nuclear receptors such as PXR. The present finding that several environmental phthalate monoesters activate mPXR, hPXR, and several hPXR variants suggests that phthalate exposure could lead to alterations in endocrine function by altering PXR-regulated steroid hormone metabolism with potential adverse health effects in exposed individuals. Urinary phthalate monoester metabolites have been reported at concentrations as high as 4 μM (MBzP) and 21 μM (M(n)BP) in a human reference population, suggesting that human exposure to phthalate monoesters is much greater than previously suspected (Blount et al., 2000) and may result in tissue concentrations in the micromolar range, that is, concentrations sufficient to activate PXR. Further investigation will be required to determine relevant phthalate tissue concentrations, whether other PXR protein variants are present and whether they contribute to individual variability in human responses to phthalates or other environmental chemicals. Future studies will explore the role of PXR and other nuclear receptors in the PPAR α -independent renal and testicular toxicities associated with exposure to DEHP and other phthalate esters.

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