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In vivo activation of the human *CYP3A4* promoter in mouse liver and regulation by pregnane X receptors

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Abstract

Human cytochrome P450 3A4 (CYP3A4) is responsible for the metabolism of numerous xenobiotics in the human liver. We have examined the activation of the human *CYP3A4* promoter in mouse liver by using *in vivo* bioluminescent imaging (BLI). Transcription of the *CYP3A4* promoter occurs as a result of a ligand binding to a nuclear orphan receptor, pregnane X receptor (PXR), followed by dimerization with another nuclear receptor, retinoid X receptor (RXR). Since this heterodimer then binds to xenobiotic response elements to activate transcription of *CYP3A4*, we examined a 13 kb promoter region of *CYP3A4* for responsiveness to dexamethasone and rifampicin. A reporter vector CYP3A4-*luc* was constructed consisting of the *CYP3A4* promoter driving the firefly luciferase gene. This DNA was injected into the tail veins of mice, and reporter gene expression was monitored in the liver region using BLI. Treatment of transfected mice with dexamethasone resulted in a 188-fold induction of luciferase, whereas treatment with rifampicin resulted in a 68-fold induction. Co-injection with a human PXR expression vector resulted in a dramatic increase in rifampicin-induced activity and a smaller increase of dexamethasone-induced activity. Co-injection of an antisense murine PXR construct with the CYP3A4-*luc* reduced both the dexamethasone- and rifampicin-induced responses, thus demonstrating that the murine PXR receptor can participate in the regulation of the human *CYP3A4* promoter in mice. The approach described here will be of general use in studying the regulation of nuclear receptors *in vivo*.

Keywords: Cytochrome P450; Pregnane X receptor; Drug metabolism; Luciferase; Imaging; *In vivo*; Gene expression

1. Introduction

The cytochrome P450 enzymes are encoded by a large family of genes (approximately 50 in humans) and are responsible for the metabolism of 90% of drugs [1–3]. Among these genes, *CYP3A4*, the most dominant P450 enzyme expressed primarily in liver and small intestine, is itself responsible for the biotransformation of 50–60% of clinical drugs and has been a focus of major research [4,5]. In addition, CYP3A4 also plays a crucial role in homeostasis of steroid hormones [6], activation of antitumor drugs and environmental procarcinogens [5], and drug–drug interactions [7,8].

The proximal promoter region of the *CYP3A4* gene has been characterized, and several binding sites for transcription factors have been identified [9]. A xenobiotic response element, an inverted repeat (IR-6, also termed an everted repeat, ER-6) separated by six nucleotides, was identified in the proximal promoter region [10,11]. More recently, a rifampicin distal enhancer module was characterized almost 8 kb from the transcriptional start site [12]. Transcriptional regulation of the CYP3A genes (*CYP3A4* in human, *Cyp3a11* in mouse) occurs as a result of ligand binding to a nuclear orphan receptor, PXR (pregnane X receptor [13]), also termed SXR (steroid and xenobiotic receptor [14]) or PAR (pregnane-activated receptor [15]). The ligand-bound PXR then dimerizes with the retinoid X receptor (RXR). The heterodimer binds to xenobiotic

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Abbreviations: BLI, bioluminescent imaging; CYP3A4, cytochrome P450 3A4; CYP3A4-*luc*, CYP3A4 promoter-luciferase reporter fusion; PXR, pregnane X receptor; and RT–PCR, reverse transcription–polymerase chain reaction.

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response elements and trans-activates transcription of the *CYP3A4* gene in a ligand-dependent manner. The molecular mechanism of PXR-mediated CYP3A4 induction implicates possible pharmaceutical applications in predicting drug metabolism and drug–drug interactions.

Due to its importance in CYP3A gene regulation, the PXR receptor has been studied extensively. The murine PXR (mPXR) was first isolated from a mouse liver library and found to be activated by synthetic pregnanes and glucocorticoids, as well as by naturally occurring steroids [16]. Subsequently, three laboratories independently identified the human PXR (hPXR) homologue [13–15]. Structurally distinct CYP3A4 inducers were demonstrated to differentially activate hPXR and mPXR in simian CV-1 cells and human Caco-2 cells using an $(ER\text{-}6)_3$-*tk*-CAT reporter or an $(ER\text{-}6)_2$-*SV40-luc* reporter [13,15]. Mobility-shift assays were used to demonstrate that the hPXR binding to the xenobiotic response element ER-6 of the *CYP3A4* promoter required heterodimerization with RXR [13–15]. The differential activation of hPXR and mPXR by xenobiotics confirmed species-specific induction of the CYP3A gene [17–19]. However, these *in vitro* PXR studies did not agree completely with some of the data observed *in vivo*. For example, it has been reported that dexamethasone induced erythromycin *N*-demethylation activity of CYP3A4 in patients [20], but did not activate the activity of hPXR in *in vitro* reporter assays [13–15]. Furthermore, *in vivo* studies showed that rifampicin strongly induces mouse Cyp3a11 mRNA and protein at a dose of 50–100 mg/kg body weight [21,22], but it did not activate mPXR *in vitro* [13–15]. Despite these discrepancies, it is believed that species-specific induction of CYP3A is determined by divergence in ligand binding domains of PXR [11,23,24]. More recently, an elegant *in vivo* study was described by Xie *et al*. [25]. These authors produced transgenic mice in which they knocked out the mouse *PXR* gene and introduced the human *PXR* gene driven by a mouse albumin promoter. These mice were able to respond more strongly to human specific inducers of the *CYP3A4* gene, as measured by induction of mouse Cyp3a11 mRNA, further suggesting that PXR determines the specificity of xenobiotic induction of CYP3A genes.

To further investigate transcriptional activation of the human *CYP3A4* promoter, we used an *in vivo* transfection system described by Liu *et al*. [26] and Zhang *et al*. [27]. This technique allows for efficient expression of exogenous genes in mouse liver by simply injecting naked plasmid into the tail vein. We designed a reporter construct consisting of a 13 kb *CYP3A4* promoter fragment driving the firefly luciferase gene and monitored the activity of the reporter using *in vivo* BLI [28,29]. This imaging method has been used successfully to both track cell growth [30–33] and monitor gene expression [34] *in vivo*, and allowed us to follow reporter activity in real-time. Our results suggested that the human *CYP3A4* promoter is functional in mouse liver and responds to both dexamethasone and rifampicin. Co-injection with a human PXR expression vector increased the responses to both dexamethasone and rifampicin, with the increased response to rifampicin being more pronounced. Co-injection with a mouse PXR expression construct increased rifampicin induction but had no significant effect on dexamethasone induction. Co-injection with an antisense mouse PXR construct led to decreasing responses to both drugs, suggesting that mPXR can activate the *CYP3A4* promoter in mouse liver cells.

2. Materials and methods

2.1. Chemicals

DMSO, dexamethasone, and rifampicin were purchased from Sigma-Aldrich.

2.2. Plasmid constructions

2.2.1. CYP3A4-luc reporter

A BAC clone containing the human *CYP3A4* promoter region was screened by PCR using primers 5′-GTTGGTACCCTGCAGTGACCACTGCCCCATCATT-G-3′ corresponding to nucleotides –1105 to –1080 and 5′-ATCAAGCTTCCTTTCAGCTCTGTGTTGCTCTTT-GC-3′ corresponding to nucleotides +40 to 69 of the *CYP3A4* promoter region (Incyte Genomics Inc.). The primers were also used to amplify a 1.2 kb promoter region of *CYP3A4* from human genomic DNA using *pfu* DNA polymerase (Stratagene). The PCR product was digested with *Kpn*I/*Hin*dIII and purified from agarose gel using a GeneClean Kit (Bio 101). The 1.2 kb promoter region was cloned into pGL3-Basic vector containing the modified firefly luciferase cDNA sequences (Promega). A 233 bp *Hin*dIII fragment containing a chimeric intron from the pCAT-3-Basic vector (Promega) was then inserted between the *CYP3A4* promoter region and the luciferase gene. A 1.88 kb *Kpn*I/*Bgl*II fragment, a 950 bp *Bgl*II fragment, and a 10 kb *Kpn*I fragment subcloned from the BAC clone were inserted sequentially into the above construct. The final construct pGL3-I-CYP3A4 (CYP3A4-*luc*) contains a 13 kb human *CYP3A4* promoter region, and this construct was used in all the experiments described below. All the joints in the construct have been confirmed by DNA sequencing (Stanford PAN Facility).

2.2.2. PXR expression vectors

The mPXR and hPXR expression vectors, pSG5-mPXR and pSG5-hPXR, were the gifts of Dr. Steven A. Kliewer and colleagues [13]. For construction of the antisense mPXR vector (pSG5-anti-mPXR), a 1.3 kb *Eco*RI fragment containing the mPXR1 cDNA from the mouse PXR expression vector pSG5-mPXR was cloned in the opposite direction with the translational stop codon adjacent to the

promoter region. The orientation of the antisense mPXR cDNA was confirmed by DNA sequencing.

2.2.3. pCyp3a11 cDNA

FvB female mice were treated intraperitoneally with 100 mg/kg of dexamethasone, and total liver RNA was extracted 8 hr later. The entire Cyp3a11 cDNA was amplified from the mouse liver RNA using a ProSTAR™ HF Single-Tube RT–PCR System (High Fidelity) Kit (Stratagene). Primer Cyp3a11TOP 5′-CTTGGTACCATGGACCTG-GTTTCAGCTCTCTCAC-3′ and primer Cyp3a11BOT 5′-CTTTCTAGATCATGCTCCAGTTATGACTGCATCC-C-3′ were used for RT–PCR. The PCR product was cloned into the pGEM3Zf(+) vector (Promega).

2.3. In vivo mouse liver cell transfection and treatments

For *in vivo* liver cell transfection, 1 μg of CYP3A4-*luc* reporter plasmid DNA was diluted in PBS buffer and injected into the tail veil of 6- to 8-week-old FvB female mice (Charles River Laboratories) within 8 sec at a volume of 1 mL/10 g of body weight. In the PXR studies, each mouse was given 5 μg of either pSG5-mPXR, pSG5-hPXR, pSG5-anti-mPXR, or pBluescript SK(+) (Stratagene) plasmid along with 1 μg of CYP3A4-*luc* reporter construct. Mice were treated with compounds 16 hr after the tail vein injection of plasmid. For each experiment, a group of three mice were treated with either of 100 μL of DMSO, 100 μL of dexamethasone solution (22 mg/mL) at 100 mg/kg body weight, or 100 μL of rifampicin solution (22 mg/mL) at 100 mg/kg body weight.

2.4. In vivo imaging

In vivo imaging was performed as described [28,34]. The substrate luciferin was injected into the intraperitoneal cavity at a dose of 150 mg/kg body weight (30 mg/mL of luciferin stock) prior to imaging. Mice were anesthetized with isoflurane/oxygen and placed on the imaging stage. Mice were imaged from the ventral side for 1 min using an *In Vivo* Imaging System (IVIS™, Xenogen) 5 min following the injection of luciferin. Relative photon emission over the liver region was quantified using LivingImage™ software (Xenogen) as an overlay on Igor image analysis software (Wavemetrics).

2.5. Northern analysis

FvB female mice were treated with DMSO, dexamethasone, or rifampicin in a group of three mice per treatment. Three age- and weight-matched females were used as a negative control. Nine hours after treatment, mouse liver tissue was excised and frozen immediately in liquid nitrogen. Total RNAs from individual liver homogenates were extracted using RNAWIZ™ Reagent (Ambion, Inc.). A single-strand antisense Cyp3a11 RNA probe was labeled using a Strip-EZ™ RNA StripAble™ RNA Probe Synthesis and Removal Kit (Ambion, Inc.). Three micrograms of total RNA from each liver homogenate was loaded in each well. The blot was hybridized and detected following the instructions of the manufacturer. After detection, the blot was stained with 0.04% methylene blue in 0.5 M sodium acetate (pH 5.2) for 20 min and destained with 25% ethanol.

3. Results

3.1. Induction of the Cyp3a11 gene in FvB mice

To test for the induction of transcription of the endogenous mouse *Cyp3a11* gene in FvB mice, we treated wild-type female mice in a group of three with DMSO, dexamethasone, or rifampicin at 100 mg/kg body weight; non-treated animals were used as a negative control. Nine hours later, total RNA from each liver was isolated. Three sets of RNA samples including non-treated, and DMSO-, dexamethasone-, and rifampicin-treated were analyzed for Cyp3a11 transcription by northern blotting. Figure 1 shows a representative data set in which Cyp3a11 mRNA was induced by both dexamethasone and rifampicin with a larger induction occurring after dexamethasone treatment. This result is consistent with previous *in vivo* studies [21,22].

3.2. Induction of the CYP3A4 promoter-luciferase reporter in mouse liver

To examine the activity of the *CYP3A4* promoter *in vivo*, plasmid DNA containing the *CYP3A4-luc* reporter gene was injected into the tail veins of FvB mice. Sixteen hours later, mice were challenged with a single dose of dexamethasone or rifampicin at 100 mg/kg body weight and

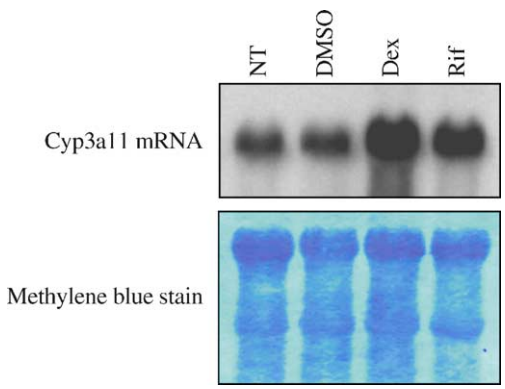

Fig. 1. Effect of dexamethasone and rifampicin on transcription of the mouse endogenous *Cyp3a11* gene in livers of FvB mice. Wild-type FvB female mice were treated with DMSO, dexamethasone (Dex), and rifampicin (Rif) at a dose of 100 mg/kg body weight. Mice were killed 9 hr following treatments. Non-treated animals (NT) were used as a negative control. Total RNA was extracted from livers, and 3 μg was analyzed by northern blot hybridization using a mouse Cyp3a11 probe. The blot was stained with methylene blue as a control for equal loading.

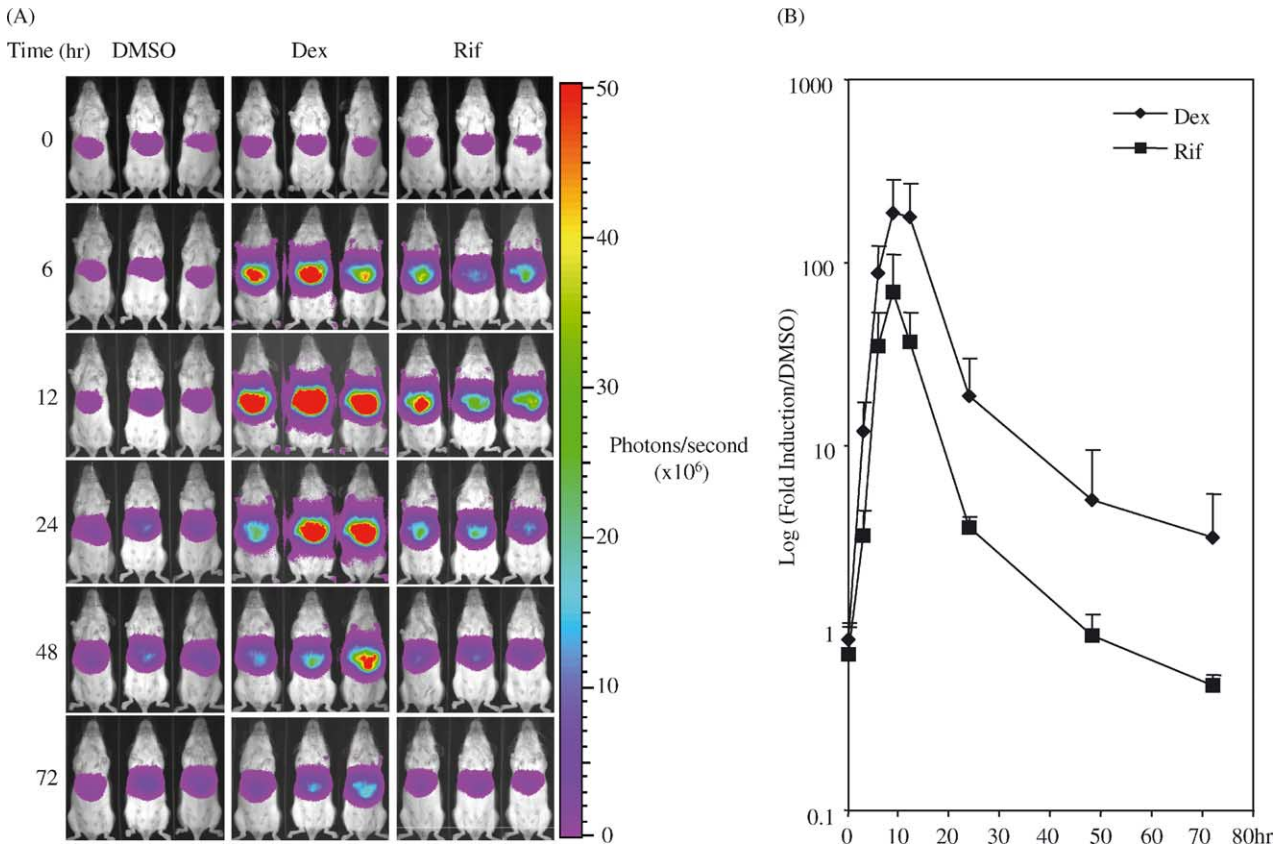

Fig. 2. Effect of dexamethasone and rifampicin on CYP3A4-*luc* expression in mouse liver. One microgram of CYP3A4-*luc* plasmid dissolved in 2.2 mL of PBS was injected rapidly into the tail veins of FvB female mice (22 g) for *in vivo* transfection. Sixteen hours after injection, mice were imaged for 1 min with high resolution at $T = 0$ (pretreatment) and then treated intraperitoneally with dexamethasone (Dex) or rifampicin (Rif) at 100 mg/kg (A). DMSO was used as the vehicle control. Luciferase signals (photons/sec) from liver regions of images were quantified using LivingImage™ software. The average fold induction was calculated by comparing to photon counts obtained from DMSO control animals (B). Values are means ± SD, N = 3.

then imaged. Pretreatment images showed that there was basal expression of the reporter gene in mouse livers (Fig. 2A). *Ex vivo* data measuring luciferase activity in a luminometer confirmed that these signals originated in the liver (data not shown). The levels of luciferase expression in the livers of mice that received the CYP3A4-*luc* construct and that were treated with dexamethasone or rifampicin were monitored by imaging over a 72-hr time course. A 188-fold induction was observed at the peak of induction in the dexamethasone-treated group (at 9 hr posttreatment) and these levels decreased to 3-fold of the basal level at 72 hr after dosing (Fig. 2A and B). Rifampicin induction peaked at 9 hr (68-fold) and diminished to the basal level by 48 hr (Fig. 2A and B).

3.3. Enhancement of the basal expression of the CYP3A4-luc reporter by hPXR and mPXR

The reporter construct CYP3A4-*luc* was co-injected with constructs encoding hPXR, mPXR, and antisense mPXR into the tail vein of FvB mice. The plasmid pBluescript SK(+) was used as an empty vector control. Sixteen hours after *in vivo* transfection, the basal level (without any drug treatment) of luciferase expression was monitored by imaging, and the *in vivo* luciferase activity from a mouse liver region was quantified (Fig. 3). Co-expression of hPXR resulted in a 23-fold increase in luciferase

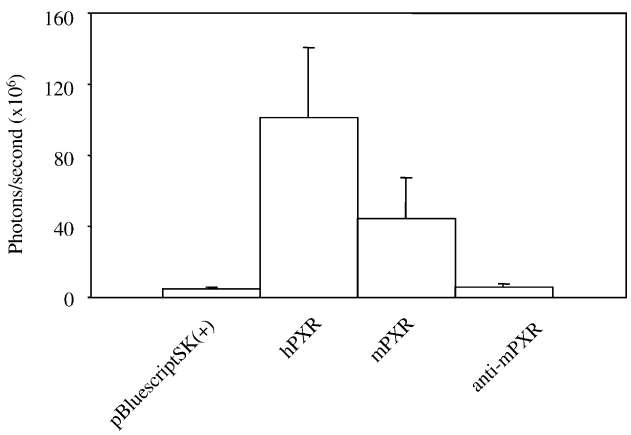

Fig. 3. Effect of nuclear receptors on basal expression of CYP3A4-*luc* in mouse liver. Five micrograms of either pSG5-hPXR (hPXR), pSG5-mPXR (mPXR), pSG5-anti-mPXR (anti-mPXR), or empty vector pBluescript SK(+) and 1 μg of the CYP3A4-*luc* plasmid were injected into the tail vein of FvB female mice. Sixteen hours after *in vivo* liver transfection, mice were imaged for 1 min with high resolution. The light signals (photons/sec) from the liver region of the images were quantified and plotted. Values are means ± SD, N = 6.

expression of CYP3A4-*luc*, while co-expression of mPXR enhanced luciferase activity 10-fold. However, introducing the antisense mPXR had little effect on the basal level of luciferase expression.

3.4. Effects of human and mouse PXRs on CYP3A4-luc induction by dexamethasone and rifampicin

In vivo transfected mice were treated with a single dose of DMSO, dexamethasone, or rifampicin (given i.p.) and imaged (Fig. 4A). Quantitative data from two independent experiments using three mice per group for each experiment are shown in panels B and C of Fig. 4. The fold induction was calculated by comparing signals to mice treated with DMSO/empty vector pBluescript SK(+), and these data are presented in Table 1. Dexamethasone and

Table 1
Summary of effects of PXRs on CYP3A4-*luc* induction in mouse liver

	Fold induction		
	DMSO	Dex	Rif
pBluescript SK(+)	1	184	35
hPXR	35	316	251
mPXR	10.6	163	52
Anti-mPXR	0.7	80	13.6

Mice treated with the combination treatments of plasmid vectors pBluescript SK(+), pSG5-hPXR (hPXR), pSG5-mPXR (mPXR), and pSG5-anti-mPXR (anti-mPXR) along with 1 μg CYP3A4-*luc* reporter construct and the drugs dexamethasone (Dex) and rifampicin (Rif) at 100 mg/mL and DMSO vehicle control were imaged 9 hr after treatment (Fig. 4A). The *in vivo* light intensities were quantified, and the average fold induction from the combination treatments of receptors and drugs was calculated by comparing to photon counts obtained from DMSO/empty vector pBluescript SK(+)-treated animals. Two independent experiments were performed using three mice per group for each experiment.

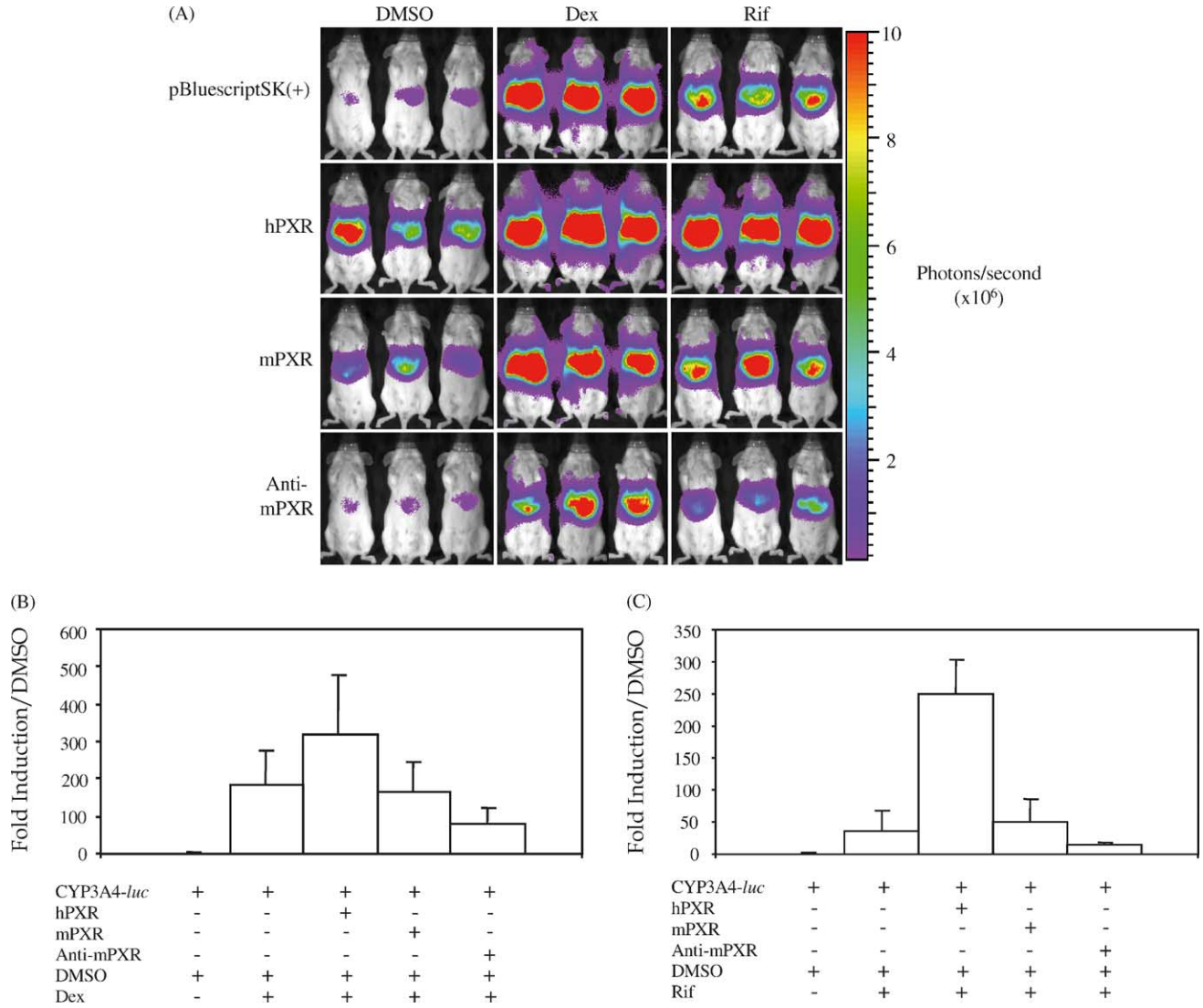

Fig. 4. Effect of nuclear receptors on CYP3A4-*luc* induction by dexamethasone and rifampicin in mice. The mice described in Fig. 3 were treated with dexamethasone (Dex) and rifampicin (Rif) at 100 mg/kg (i.p.); DMSO was used as the vehicle control. Mice were imaged 9 hr after treatment (A). The *in vivo* light intensities were quantified, and the average fold induction from the combination treatments of receptors and drugs was calculated by comparing to photon counts obtained from animals treated with DMSO/empty vector pBluescript SK(+) (B and C). Values are means ± SD, N = 6.

rifampicin induced expression of the CYP3A4-*luc* 184- and 35-fold, respectively. When the hPXR construct was co-expressed along with the reporter construct, we noted a dramatic increase in luciferase induction by rifampicin (617%; from 35- to 251-fold). Dexamethasone induction was increased by 72% (from 184- to 316-fold). Expression of exogenous mPXR had a minimal effect on the dexamethasone induction of CYP3A4-*luc*, while rifampicin induction was increased by 48% (from 35- to 52-fold). When antisense mPXR construct was co-injected, dexamethasone induction was reduced by 57% (from 184- to 80-fold) and rifampicin induction was decreased by 61% (from 35- to 13.6-fold).

4. Discussion

In this study, we combined *in vivo* BLI with an efficient *in vivo* transfection method for rapid analysis of the response of human CYP3A4 to selected drugs in living animals. The CYP3A4-*luc* reporter construct contained 13 kb of 5′ sequence including the proximal regulatory element and distal rifampicin enhancer module of CYP3A4 [9,12]. Use of *in vivo* BLI allowed us to examine gene regulation in living animals, and to perform temporal analyses of gene expression. Induction of our CYP3A4-*luc* reporter by dexamethasone was almost 3-fold greater than that observed with rifampicin (Fig. 2B). Northern analyses also showed that dexamethasone induced the endogenous mouse Cyp3a11 mRNA over the level induced by rifampicin (Fig. 1). The induction patterns of CYP3A4-*luc* reporter correlated with that of the mouse homologue *Cyp3a11* gene. Our data demonstrate that in the absence of the *hPXR* gene the human *CYP3A4* promoter behaves similarly to the mouse *Cyp3a11* promoter in mouse liver with respect to induction by dexamethasone and rifampicin. This is consistent with the notion that the host cellular environment is the major determinant of CYP3A expression [11]. The differences in dexamethasone and rifampicin induction could have been due to differential activation of the mouse PXR receptor by these two drugs.

It is interesting that basal expression of the reporter construct was enhanced markedly when either human or mouse PXR was introduced into mouse livers (Fig. 3). It is possible that natural mPXR ligands such as steroids activate the exogenous PXR, leading to an increase of basal expression of the reporter gene. We also noted that enhancement of the basal luciferase expression by hPXR was stronger than that by mPXR (Fig. 3). It is possible that the human PXR-mRXRα heterodimer has higher binding affinity for the *CYP3A4* promoter than does the mouse PXR-mRXRα. A second possibility is that some mouse endogenous ligands are better activators of hPXR than mPXR.

Co-expression of hPXR resulted in a dramatic enhancement of transgene induction by rifampicin such that differences between rifampicin and dexamethasone induction of CYP3A4-*luc* were abolished (Fig. 4B and C, and Table 1). This suggests that rifampicin is a better trans-activator of hPXR than of mPXR and is in agreement with previous studies [13,23]. Expression of exogenous mPXR had a minimal effect on the dexamethasone induction of the CYP3A4-*luc* reporter (Fig. 4). This result implied that the endogenous mPXR may have been sufficient to mediate dexamethasone induction of CYP3A4-*luc*. However, mPXR increased rifampicin induction of CYP3A4-*luc* by 48%, suggesting that an increased mPXR receptor can further enhance activity of a weak activator like rifampicin. When the antisense mPXR construct was introduced, drug induction of CYP3A4-*luc* was reduced (Fig. 4 and Table 1), further demonstrating that mPXR is involved in CYP3A4-*luc* induction in mouse livers by both dexamethasone and rifampicin. Previous *in vitro* studies showed that rifampicin did not activate mPXR [8,13,23]. However, all of these studies used a reporter consisting of multiple copies of the ER-6 response element of the human *CYP3A4* gene in their constructs, and experiments were performed in cell lines. It is possible that activation of mPXR by rifampicin requires an additional response element(s) for optimal activation of *CYP3A4* promoter. We used the relatively larger native 13 kb promoter in the reporter construct that contained at least one additional rifampicin response module [12]. It is also possible that some transcription factors required for trans-activation were missing in the cell lines used in the previous studies, or it is possible that the *in vivo* assay used in our studies overcomes these limitations.

Since dexamethasone activated mPXR to a higher level than rifampicin in mice, as measured by induction of Cyp3a11 mRNA and CYP3A4-*luc* reporter (Figs. 1 and 2), one might expect that the antisense mPXR treatment would cause a greater reduction in dexamethasone induction versus rifampicin induction. However, our results showed that the reduction of dexamethasone and rifampicin induction by antisense mPXR was comparable (57 and 61%, respectively). This result suggests that there may be other mechanisms involved in dexamethasone induction of the *CYP3A4* gene. Indeed, it was demonstrated that the glucocorticoid receptor was required for steroid induction of human CYP3A4 using another method [35].

In summary, we have demonstrated that the human *CYP3A4* promoter was functional in mouse liver and could be induced by both dexamethasone and rifampicin. Regulation of the *CYP3A4* promoter in mice mimics that of the endogenous *Cyp3a11* gene. Co- expression of the *hPXR* gene significantly increased the induction of our CYP3A4-*luc* construct by rifampicin. We also further demonstrated that mPXR is involved in CYP3A4 induction in mice by dexamethasone and rifampicin.

Note added in proof

While this manuscript was in review, Schuetz *et al.* (Schuetz E, Lan L, Yasuda K, Kim R, Kocarek TA, Schuetz

J, Strom S. Development of a real-time in vivo transcription assay: application reveals pregnane X receptor-mediated induction of CYP3A4 by cancer chemotherapeutic agents. Mol Pharmacol 2002;62:439–45) also reported the use of hydrodynamic transfection and BLI to detect the induction of CYP3A4.

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