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Repression of PXR-mediated induction of hepatic *CYP3A* gene expression by protein kinase C

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Abstract

Pregnane X receptor (PXR, NR1I2) regulates the inducible expression of the 3A sub-family of cytochrome P450 genes (*CYP3A*). CYP3A enzymes are responsible for the oxidative metabolism of a wide array of endobiotic and xenobiotic compounds. Hepatic *CYP3A* gene expression is rapidly down-regulated during inflammation and sepsis. There are twelve protein kinase C (PKC) isoforms, classified into three subfamilies according to the structure of the N-terminal regulatory domain and their sensitivity to calcium and diacylglycerol. It is now well accepted that cytokine stimulation of hepatocytes increases intracellular PKC activity during inflammation and sepsis. We show here that protein kinase C alpha (PKCα) and phorbol ester-dependent PKC signaling dramatically repressed PXR activity in both, cell-based reporter gene assays and in hepatocytes. Moreover, treatment with the protein phosphatase PP1/PP2A inhibitor okadaic acid (OA) totally abolished PXR activity in reporter gene assays and in cultured hepatocytes. In mammalian two-hybrid assays, treatment with phorbol 12-myristate 13-acetate (PMA) increased the strength of interaction between PXR and the nuclear receptor co-repressor protein (NCoR). Treatment with PMA also abolished the ligand-dependent interaction between PXR and the steroid receptor co-activator 1 protein (SRC1). Our findings suggest that activation of the protein kinase C signaling pathway represses PXR activity through alterations in PXR-protein co-factor complexes, possibly through direct alterations in the phosphorylation status of one or all of these proteins. In addition, our data potentially provide important insights into the molecular mechanism of the repression of hepatic *CYP3A* gene expression that occurs during the inflammatory response.
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1. Introduction

Protein kinase C (PKC) plays a key role in both the production of and the response to cytokine signaling in liver. Release of pro-inflammatory cytokines [interleukin-1, interleukin-6, and tumor necrosis factor-alpha (TNF-α)] from Kupffer cells is dependent upon PKC activity [1]. During sepsis and inflammation, cytokine-mediated stimulation of hepatocytes rapidly initiates PKC-dependent

intracellular signaling pathways (reviewed in [2]). One of the most important responses following release of pro-inflammatory cytokines is the rapid repression of cytochrome P-450 (*CYP*) gene expression in liver [3,4]. It is now recognized that in post-operative patients the down-regulation of hepatic *CYP* gene expression and activity has a major impact on the disposition of prescription medications [5]. This is especially true of *CYP3A4* gene expression, since the CYP3A4 enzyme catalyzes the oxidative metabolism of over 50% of all clinically prescribed drugs [6].

Most, if not all, nuclear receptor superfamily members exist as phosphoproteins and their phosphorylation is dynamically regulated [7]. Additionally, many nuclear receptors are regulated by PKC including the glucocorticoid receptor [8], the progesterone receptor [9], estrogen receptor [10], the thyroid receptor [11], and the vitamin D receptor [12]. Notably, PKC-mediated phosphorylation

of the vitamin D receptor modulates *CYP3A4* gene expression in an intestinal cell line [13]. Thus, PKC appears to play a pivotal role in regulating the activity of multiple nuclear receptors. The nuclear receptor pregnane X receptor (PXR, NR1I2) regulates the inducible expression of hepatic CYP3A family members in rodents and humans [14–17]. While much is known regarding the identity of PXR-target genes and PXR ligands, little is known regarding the potential for PKC-mediated regulation of PXR activity in liver cells.

The best-characterized hepatic PXR-target promoter in humans is *CYP3A4*. The *CYP3A4* promoter has two PXR/RXR heterodimer binding sites [18]. One site is located approximately 170 bp upstream of the transcription initiation site, while the other binding site is located approximately 8 kb upstream. The removal of the intervening DNA sequences places these two elements in tandem and produces a highly PXR-responsive xenobiotic-responsive enhancer module (XREM). This *CYP3A4* promoter has been placed upstream of the luciferase reporter gene and is termed XREM-Luc [18]. The XREM-Luc reporter gene has been subsequently used to assay the activation of PXR using transient transfection analysis in CV-1 cells [18,19]. Primary hepatocytes also serve as a useful model to study modulation of PXR activity [20].

We show here that over-expression of a constitutively active form of PKCα, called PKC7, represses PXR activity in cell-based XREM-Luc reporter gene assays. Additionally, we show that activation of PKC signaling using phorbol 12-myristate 13-acetate (PMA) represses PXR activity in cell-based reporter gene assays and in cultured hepatocytes, while treatment with the PKC-inactive phorbol ester 4α-Phorbol 12-myristate 13-acetate (4α-PMA) does not. Moreover, treatment of PXR-transfected CV-1 cells and cultured primary hepatocytes with the protein phosphatase PP1/2A inhibitor okadaic acid (OA) totally abolishes ligand-dependent PXR activity. Finally, mammalian two-hybrid analysis reveals that treatment with PMA increases the strength of interaction between PXR and NCoR, and also inhibits ligand-dependent interaction between PXR and SRC1, while treatment with the PKC-inactive phorbol ester 4α-PMA does not.

Hepatic PKC signaling is rapidly elevated following cytokine-mediated stimulation and these data provide important insights into the molecular mechanism of inflammation- and sepsis-mediated down-regulation of hepatic *CYP3A* gene expression. Our findings suggest that activation of the protein kinase C signaling pathway represses PXR activity through alterations in the PXR-co-factor protein complex, likely through direct alterations in the phosphorylation status of one or more of these proteins. Future studies will be focused on identifying and comparing potential sites of PXR, Nuclear receptor co-repressor protein (NCoR), and Steroid receptor co-activator 1 protein (SRC1) phosphorylation following cytokine stimulation and activation of the PKC signaling pathway.

2. Materials and methods

2.1. Plasmids and chemicals

The full-length human PXR and mouse PXR mammalian expression vectors were described previously [16,17]. The mammalian expression vector encoding the constitutively active form of PKCα (PKC7) was described previously [21]. XREM-LUC was described previously [18]. VP16-mPXR encodes the full-length mouse PXR fused to VP16. GAL-NCoR and GAL-SRC1 were generous gifts from Dr. Barry Forman [22]. The pCMV-β-Gal and pSV-β-Gal plasmids are commercially available (Invitrogen). All compounds were purchased from Sigma and dissolved as 1000X stocks in DMSO.

2.2. Cell culture and transient transfection of CV-1 cells

The XREM-LUC reporter gene assays were performed as described [23]. The mammalian two-hybrid system analysis was performed as described [24]. Transfection mixtures contained pFR-LUC (20 ng), GAL4-co-factor (20 ng), VP16-mPXR (10 ng), PKC7 (5 ng), pSV-β-GAL (40 ng), and pBluescript (20 ng). Twenty-four hour post-transfection, cells were drug-treated for 24 h. The luciferase and β-galactosidase activities were determined using the dual-light kit as per the manufacturer's instructions (Applied Biosystems).

2.3. Northern blot analysis

Mouse hepatocytes were isolated from male mice as described [20] and plated in 6-well collagen-coated plates at 8×10^5 cells/well in DME Medium supplemented with 100 nM dexamethasone, 5% FBS, 1 μM Insulin, 2 mM L-glutamine, 100 units/ml penicillin and 100 μg/ml streptomycin. Twelve to sixteen hours after plating, the medium was replaced with maintenance medium (William's E medium supplemented with 100 nM dexamethasone, 100 units/ml penicillin, 100 μg/ml streptomycin and 1x Insulin–Transferrin–Selenium (Invitrogen)). The medium was replaced every 24 h. Ninety hour post-plating the hepatocytes were treated with drugs in maintenance medium for 24 h. Total RNA was isolated using Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA (10 μg/lane) was resolved on 3.7 formaldehyde 1% agarose gel for Northern blot analysis. Blots were hybridized with ^{32}P-labeled cDNA corresponding to the cDNA sequence for mouse Cyp3a11 (bases 69 to 1609, GenBank NM007818). The 18S ribosomal RNA probe was amplified from liver cDNA using commercially available primers per the manufacturer's instruction (Ambion).

2.4. Transfection of mouse hepatocytes

Primary mouse hepatocytes were plated at a density of 4×10^5 cells/well in 12-well collagen-coated plates and cultured for 48 h before transfection. Transfection mixtures contained XREM-Luc (200 ng), pCMVβ-Gal (400 ng) and pBluescript (400 ng). Hepatocytes were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction with only minor modification. Briefly, for each well, 3 μl of Lipofectamine 2000 was mixed with 100 μl of OPTIMEM (Invitrogen) and incubated at room temperature for 5 min. One microgram of DNA was added to the Lipofectamine 2000/OPTIMEM mixture and incubated at room temperature for an additional 20 min. Hepatocytes were rinsed with 300 μl OPTIMEM once and the medium was then replaced with 500 μl of OPTIMEM per well. The Lipofectamine 2000/OPTIMEM/DNA mixture was then added drop-wise to each well. After incubation overnight, the medium was replaced with 1 ml of hepatocyte-maintenance medium containing drugs. Twenty-four hour post-drug treatment, hepatocytes were lysed and the reporter gene assays were performed as described [23].

3. Results

3.1. Functional regulation of PXR activity by PKCα signaling in CV-1 cells

To investigate the functional regulation of PXR activity by PKCα-mediated signaling, we performed a series of reporter gene assays in CV-1 cells. The synthetic steroid pregnenolone 16α-carbonitrile (PCN) is a specific rodent PXR agonist. Co-transfection of increasing amounts of plasmid DNA encoding the constitutively active form of PKC-α, called PKC7, repressed the induction of reporter gene activity by PCN in a dose-dependent manner (Fig. 1).

Because CV-1 cells express significant levels of PKCα [25], we next performed the XREM-Luc reporter gene assay in PXR-transfected CV-1 cells and treated with increasing concentrations of PMA (1, 10, and 100 nM) and PKC-inactive 4α-PMA (1, 10, and 100 nM). Treatment with PMA repressed the induction of XREM-Luc reporter gene activity in a dose-dependent manner, while treatment with 4α-PMA did not (Fig. 2). We also transfected CV-1 cells with the expression vector encoding VP16-PXR together with the XREM-Luc reporter gene and treated with PMA (100 nM) and 4α-PMA (100 nM). Treatment with PMA repressed the constitutive activity of VP16-PXR, while treatment with 4α-PMA did not (Fig. 2).

Similar to what has been previously shown for the constitutive androstane receptor [26], we hypothesized that inhibition of the protein phosphatases PP1/PP2A may repress PXR activity. Treatment of CV-1 cells with the potent PP1/PP2A inhibitor okadaic acid (OA) produced
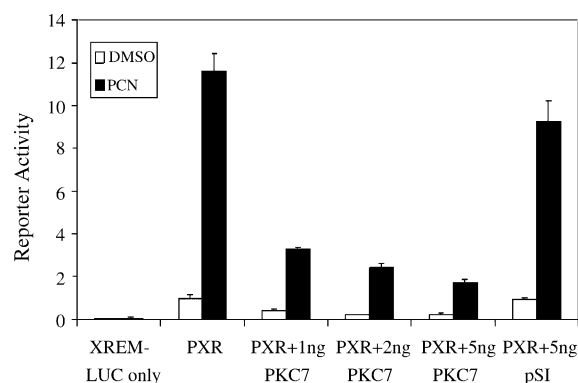

Fig. 1. Constitutively active PKCα signaling (PKC7) represses PXR-dependent XREM-Luc reporter gene activity in CV-1 cells. Transient transfection was performed as described in materials and methods. Twenty-four hour post-transfection, CV-1 cells were treated with vehicle (0.1% DMSO) and 10 μM PCN for 24 h in the presence of the indicated amounts of the PKC7 expression vector or empty vector pSI. The data represent the mean of replicates ± S.E.M. ($n = 4$) and are expressed as reporter gene activity.

striking repression of ligand-dependent PXR activity in the XREM-Luc reporter gene assay (Fig. 3).

3.2. PKC signaling represses the activity of endogenous PXR in hepatocytes

To determine whether PKC signaling represses endogenous PXR activity in liver cells, we performed northern blot analyses with RNA isolated from cultured primary mouse hepatocytes. The *Cyp3a11* gene represents the prototypical PXR-target gene in mice [27]. Ninety hour post-plating, hepatocytes were treated with PMA (300 nM), 4α- PMA (300 nM), and OA (100 nM). Treatment of hepatocytes with either PMA or OA repressed the PCN-mediated induction of *Cyp3a11* gene expression in
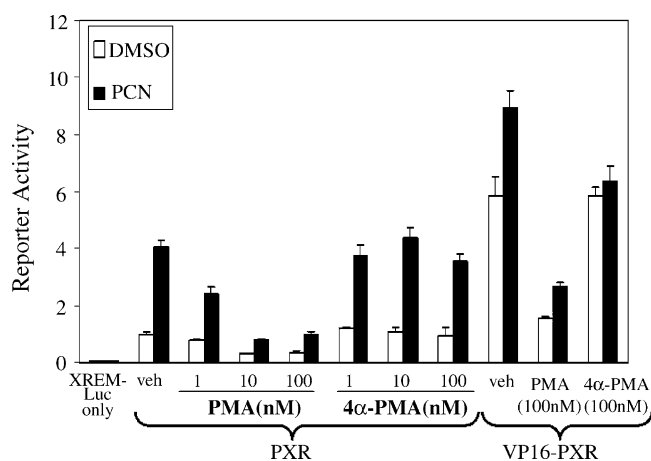

Fig. 2. Activation of endogenous phorbol ester-dependent PKC signaling represses XREM-Luc reporter gene activity in CV-1 cells. Twenty-four hour post-transfection, CV-1 cells were treated with the indicated concentrations (nM) of PMA or PKC-inactive 4-α PMA in the presence and absence of PCN (10 μM) for 24 h. The data represent the mean of replicates ± S.E.M. ($n = 4$) and are expressed as reporter gene activity.
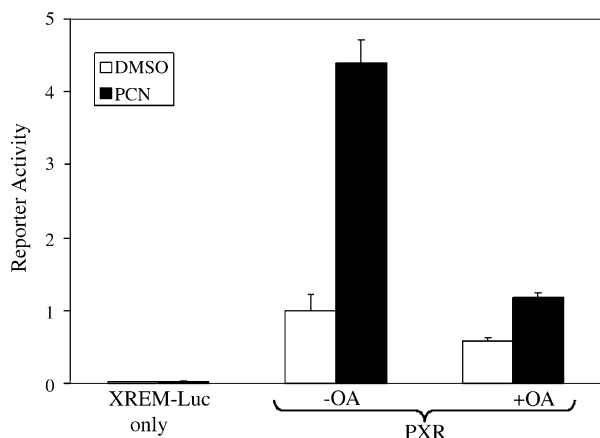
Fig. 3. Inhibition of protein phosphatases PP1/PP2A with okadaic acid represses XREM-Luc reporter gene activity in CV-1 cells. Twenty-four hour post-transfection, CV-1 cells were treated with PCN (10 μM) in the presence and absence of 100 nM okadaic acid (OA) for 24 h. The data represent the mean of replicates ± S.E.M. ($n = 4$) and are normalized against β-galactosidase activity and are expressed as reporter gene activity.
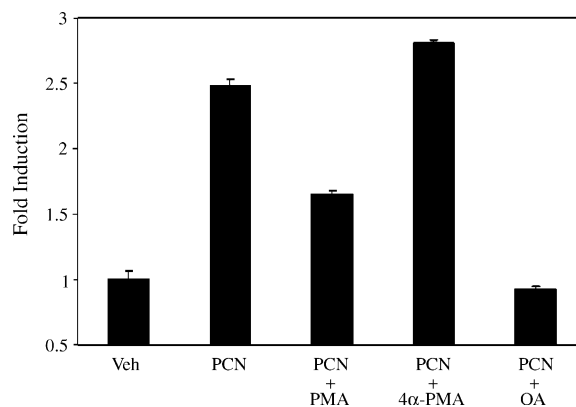

Fig. 5. Treatment with PMA and OA represses endogenous PXR activity in primary hepatocytes. Mouse hepatocytes were transfected with 200 ng of XREM-LUC and 400 ng of pCMV-β-Gal expression vectors in 12-well plates. Twenty-four hour post-transfection, cells were treated with vehicle (Veh, 0.1% DMSO), 10 μM PCN or 10 μM PCN in combination with 300 nM PMA, 300 nM 4 α-PMA or 100 nM OA for 24 h. The data represent the mean of assays performed in triplicate ± S.E.M. and is normalized to β-galactosidase activity and is expressed as fold induction over vehicle control.

hepatocytes, while treatment with 4α-PMA did not (Fig. 4). Treatment of hepatocytes with as little as 10 nM OA dramatically repressed the induction of *Cyp3a11* gene expression by 10 μM PCN, while higher concentrations (20, 50, and 100 nM) totally abolished PCN-mediated *Cyp3a11* gene expression (Fig. 4).

To examine the activity of endogenous PXR in the context of a hepatocyte more closely, we transfected primary mouse hepatocytes with the XREM-Luc reporter gene and treated with PMA (300 nM), 4α-PMA (300 nM), or OA (100 nM) (Fig. 5). Treatment with PCN alone induced the expression of the XREM-LUC reporter gene. Co-treatment with PMA repressed XREM-Luc reporter gene activity, while co-treatment with 4α-PMA did not. Additionally, co-treatment with OA produced dramatic

repression of XREM-Luc reporter gene activity in transfected hepatocytes (Fig. 5).

3.3. Effect of PKC signaling on PXR-protein co-factor interactions

The interaction between nuclear receptor co-repressor proteins and PXR has been reported to repress PXR activity in cells [28]. Because we observed repression of PXR activity following activation of the PKC signaling pathway, we sought to determine whether pharmacological activation of this pathway would alter the strength of PXR-protein co-factor interactions using the mammalian two-hybrid system. CV-1 cells were transfected with the expression vector encoding the receptor interacting domain
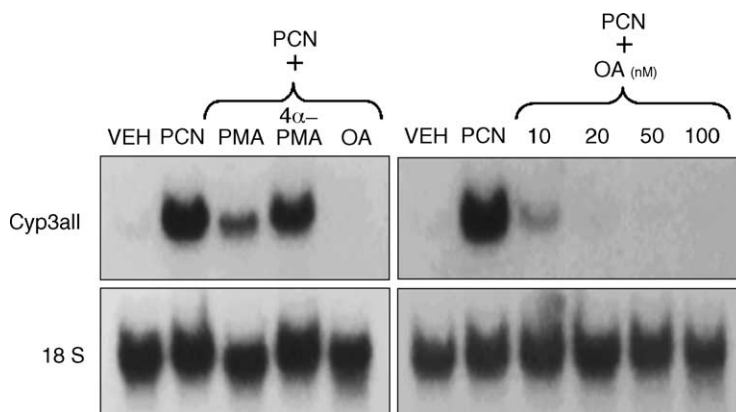

Fig. 4. Treatment with PMA and okadaic acid represses PXR-mediated induction of Cyp3a11 gene expression in cultured primary hepatocytes. Mouse hepatocytes were cultured in 6-well plates for 90 h. Hepatocytes were treated with vehicle (0.1% DMSO), 10 μM PCN, 10 μM PCN plus 300 nM PMA, 10 μM PCN plus 300 nM 4 α-PMA, or 100 nM OA. Hepatocytes were also treated with 10 μM PCN plus the indicated concentrations of okadaic acid for 24 h. Total RNA was isolated and 10 μg per lane was resolved on 3.7% formaldehyde 1% agarose gel. Following transfer to positively charged nylon membrane, Northern blot analyses were performed using cDNA probes for mouse Cyp3a11 and 18S ribosomal RNA. Shown are typical results from at least three independent experiments.
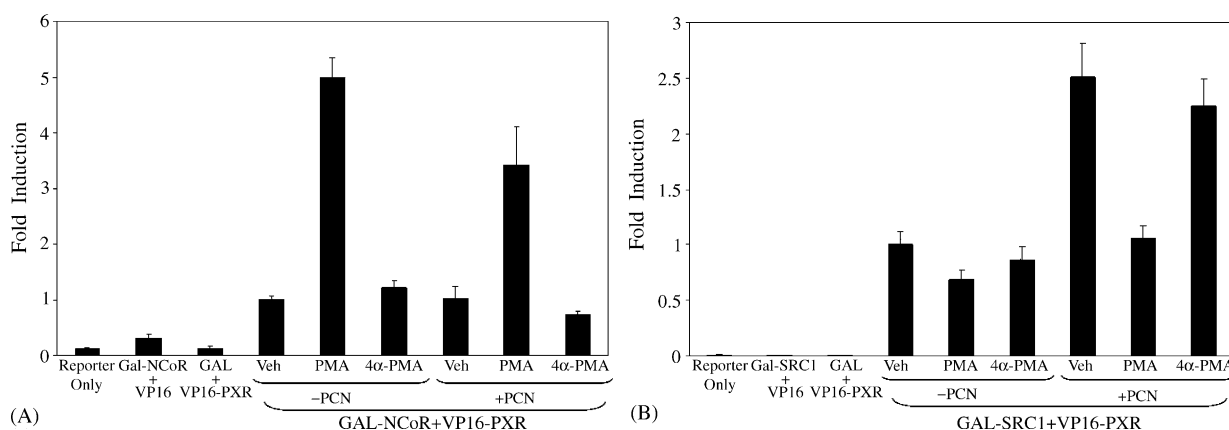
Fig. 6. Activation of PKC signaling alters the interaction between PXR and co-factors in CV-1 Cells. CV-1 cells were transfected with VP16-PXR together with either (A) GAL-NCoR or (B) GAL-SRC1 and the pFR-Luc reporter gene as described in materials and methods. Twenty-four hour post-transfection, CV-1 cells were treated with vehicle (Veh, 0.1% DMSO), 100 nM PMA or 100 nM 4α-PMA in the absence or presence of 10 μm PCN for 24 h before assays. The data represent the mean ± S.E.M. ($n = 4$).

from either the nuclear receptor co-repressor protein NCoR or the co-activator protein SRC1 fused to GAL4 DNA-binding domain together with the VP16-tagged mouse PXR and the GAL4-responsive luciferase reporter gene *pFR-Luc*. To activate the PKC signaling pathway we used the PKC activator PMA (100 nM). The PKC-inactive 4α-PMA (100 nM) was used as a negative control. As shown in Fig. 6A, treatment with PMA strengthened the interaction between PXR and NCoR in the absence as well as in the presence of PCN, while treatment with the PKC-inactive 4α-PMA did not (Fig. 6A). Conversely, co-treatment of transfected cells with PMA and PCN inhibited the ligand-dependent interaction between PXR and SRC1, while treatment with 4α-PMA did not produce this effect (Fig. 6B).

4. Discussion

It has been known for years that the expression of cytochrome P450 genes is down regulated by various inflammatory stimuli such as bacterial infection or inflammation (reviewed in [29]). For example, acute inflammation after elective surgery is associated with a significant decline in the CYP3A4 activity [5]. Although the molecular mechanism of the down-regulation is still currently unknown, much of the effect is attributed to the release of cytokines such as interleukin-1, interleukin-6, and TNF-α from Kupffer cells (reviewed in [2]). Interleukin-1, inter-leukin-6 and tumor necrosis factor-α all activate the protein kinase C signaling in hepatocytes (reviewed in [2]).

Here we show for the first time that the activity of PXR can be regulated by the activation of PKC-α. The data presented here suggest that alterations in the phosphorylation status of PXR and/or PXR-interacting proteins following activation of the PKC signaling pathway likely

participate in the observed down-regulation of CYPs during inflammation.

Similar to these studies, repression of vitamin D receptor activity following PKC activation towards *CYP3A4* gene expression has been demonstrated using a human intestinal cell line [12]. Our data reveal that PXR activity is repressed following activation of the PKC signaling pathway in hepatocytes. Repression of the transcriptional activity of the VP16-PXR fusion protein by PMA indicates that regulation of PXR by PKC-α may prevent it from binding to its target DNA sequences, or perhaps by modulating interaction with protein co-factors that mediate transre-pression and transactivation. This is consistent with the fact that nuclear receptor proteins are phosphorylated mainly in their N-terminal AF-1, C-terminal AF-2, and DNA-binding domains [7].

The nuclear receptor co-repressor protein, NCoR, was originally identified in a yeast two-hybrid protein–protein interaction screen using non-liganded thyroid hormone receptor as bait [30]. In addition to the thyroid hormone receptor, NCoR associates with other nuclear receptor proteins including the retinoic acid receptor, the estrogen receptor, and PXR [22,31]. Recruitment of NCoR to nuclear receptors represses their activity by recruiting a multi-protein complex that contains histone deacetylase activity [32]. Our data show that the PXR-NCoR interaction is strengthened following activation of PKCα signaling. Moreover, both inhibition of protein phosphatases PP1/PP2A by OA treatment and co-expression of PKC7 strengthened the interaction between PXR and NCoR (data not shown). A similar phenomenon is observed with an NCoR-related protein called the silencing mediator for retinoid and thyroid receptor protein [33]. Activation of the theta isoform of PKC recruits the silencing mediator for retinoid and thyroid receptor co-repressor protein to the thyroid hormone, 9-cis-retinoic acid, and the retinoic acid receptors. Our data also suggest that recruitment of the coactivator proteins, such as SRC1, is impaired follow-

ing activation of PKC signaling pathway. In addition to recruitment of protein co-factors, the transcriptional activity of nuclear receptors may also be affected by (1) protein degradation; (2) alterations in affinity for DNA-response elements; (3) alterations in protein sub-cellular localization; and (4) alterations in gene expression. In CV-1 cells, co-transfection of PKC7 did not change the protein level of PXR (data not shown) suggesting that it is not likely that PKC activation promotes degradation of the PXR protein. Future studies will use electrophoretic mobility shift assays to determine whether increased PKC signaling produces alterations in DNA-binding by PXR. Additional studies, using GFP-tagged PXR proteins, will determine whether PKC signaling produces alterations in PXR's sub-cellular location. In light of the recent work that demonstrates that IL-6 decreases the expression of PXR genes [34], PKC signaling may also inhibit CYP3A activity through down-regulation of PXR expression in hepatocytes. This possibility is currently under investigation.

In summary, our findings show that activation of the PKC signaling pathway represses the ability of PXR to transactivate its target genes in both cell-based biochemical assays as well as in cultured hepatocytes. Moreover, our data suggest that the molecular basis of sepsis- and inflammatory-based repression of CYP3A gene expression occurs through activation of the protein kinase C signaling pathway which then represses PXR activity by producing alterations in the PXR-protein co-factor complex. The possibility exists that this occurs through direct alterations in the phosphorylation status of PXR, NCoR, SRC1 or all of these proteins. Future studies will determine whether the potential sites of serine–threonine phosphorylation in these proteins are modified following stimulation with pro-inflammatory cytokines and activation of the PKC signal transduction pathway.

Acknowledgement

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