REGULATION OF THE PREGNANE X RECEPTOR SIGNALING PATHWAY

By

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Abstract

Liver-enriched nuclear receptors (NRs) collectively function as metabolic and toxicological 'sensors' that mediate liver-specific gene-activation in mammals. NR-mediated gene-environment interaction regulates important steps in the hepatic uptake, metabolism and excretion of glucose, fatty acids, lipoproteins, cholesterol, bile acids, and xenobiotics. While it is well-recognized that ligand-binding is the primary mechanism behind activation of NRs, recent research is revealing that multiple signal transduction pathways modulate NR-function in liver. The interface between specific signal transduction pathways and NRs helps to determine their overall responsiveness to various environmental and physiological stimuli. The pregnane x receptor (PXR, NR1I2) was identified in 1998 as a member of the NR superfamily of ligand-activated transcription factors. PXR is activated by a broad range of lipophilic compounds in a species-specific manner. The primary function ascribed to PXR is the homeostatic control of steroids, bile acids, and xenobiotics. This function is mediated through PXR's ability to coordinately activate gene expression and regulate the subsequent activity of phase I and phase II metabolic enzymes, as well as several membrane transporter proteins. While PXR likely evolved primarily to protect the liver from toxic assault, its activation also represents the molecular basis for an important class of drug-drug, herb-drug, and food-drug interactions. While ligand binding is the primary mode of PXR activation, several signal transduction pathways interface with the PXR protein to determine its overall responsiveness to environmental stimuli. Multiple signaling pathways modulate the

activity of PXR, likely through direct alteration of the phosphorylation status of the receptor and its protein cofactors. Therefore, specific combinations of ligand binding and cell signaling pathways affect PXR-mediated gene activation and determine the overall biological response.

This dissertation contributes to the molecular understanding of the regulation of PXR by novel agonists, cAMP-dependent protein kinase (PKA) signaling, and phosphorylation. The results presented here were primarily obtained from mouse and tissue culture systems. This dissertation identifies Tian Xian, a traditional Chinese herbal anti-cancer remedy, as a novel PXR activator. This evidence suggests that Tian Xian should be used cautiously by cancer patients taking chemotherapy due to its potential to increase the metabolism of co-administered medications. In addition, data presented here show that activation of PKA signaling modulates PXR activity in a species-specific manner. It is further revealed that PXR exists as phospho-protein *in vivo* and that the activation of PKA signaling modulates the phospho-threonine status of PXR. Finally, the potential phosphorylation sites within the PXR protein are identified. These phosphorylation sites are characterized, using a phosphomimetic and phospho-deficient site-directed mutagenesis based approach, based on their ability to modulate PXR activity. Taken together, the work presented in this dissertation contributes to understanding the interface between ligands, signal transduction pathways and PXR activity, which is critical for the development of safe and effective therapeutic strategies.

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List of Abbreviations

ABCA1: ATP Binding Cassette Transporter A1

AF-1: Activation Function 1

AF-2: Activation Function 2

ALAS: Aminolevulinic Acid Synthase

ALR: Augmenter of Liver Regeneration

ALT: Alanine Aminotransferase

AMPK: AMP-activated Protein Kinase

APAP: Acetaminophen

AR: Androgen Receptor

BAC: Bacterial Artificial Chromosome

BXR: Benzoate X Receptor

CA: Cholic Acid

CAR: Constitutive Androstane Receptor

CARLA: Co-activator Receptor Ligand Assay

CBP: CREB Binding Protein

CCRP: Cytoplasmic CAR Retention Protein

CDCA: Chenodeoxycholic Acid

CDK2: Cyclin-dependent Kinase 2

CK2: Casein Kinase 2

COUP-TF: Chicken Ovalbumin Upstream Promoter Transcription Factor

COX2: Cyclooxygenase 2

CPT1A: Carnitine Palmitoyltransferase 1A

CREB: Cyclic AMP Response Element Binding Protein

CYP: Cytochrome P450

DBD: DNA-binding Domain

DSS: Dextran Sulfate Sodium

EGF: Epidermal Growth Factor

ER: Estrogen Receptor

ERK: Extracellular Regulated Kinase

FGF: Fibroblast Growth Factor

FGFR4: FGF Receptor 4

FOXO1/A2: Forkhead Box Transcription Factor O1/A2

FXR: Farnesoid X Receptor

G6Pase: Glucose 6 Phosphatase

G6PDH: Glucose 6 Phosphate Dehydrogenase

GPCR: G-protein Coupled Receptor

GR: Glucocorticoid Receptor

GST: Glutathione S Transferase

H: Hinge Region

HAT: Histone Acetyl Transferase

HDAC: Histone Deacetylase

HDL: High Density Lipoprotein

HGF: Hepatocyte Growth Factor

HMGCS2: 3-Hydroxy-3-Methylglutarate-CoA Synthase 2

HNF4α: Hepatocyte Nuclear Factor 4 Alpha

HSP90: Heat Shock Protein 90

IBD: Inflammatory Bowel Disease

ICAM-1: Intercellular Adhesion Molecule 1

IFNα: Interferon Alpha

IGF: Insulin-like Growth Factor

IL: Interleukin

iNOS: Inducible Nitric Oxide Synthase

JNK: Jun-kinase

LBD: Ligand-binding Domain

LCA: Lithocholic Acid

LPS: Lipopolysaccharide

LRH-1: Liver Receptor Homolog 1

LXR: Liver X Receptor

MAPK: Mitogen-activated Protein Kinase

MDR1: Multi-drug Resistance 1

MRP2/3: Multi-drug Resistance Associated Protein 2/3

NAPQI: N-acetyl-p-benzoquinone Imine

NCoR: Nuclear Receptor Co-repressor

NFκB: Nuclear Transcription Factor Kappa B

NLS: Nuclear Localization Signal

NR: Nuclear Receptor

OATP2: Organic Ion Transporting Protein 2

PB: Phenobarbital

PBMC: Peripheral Blood Mononuclear Cell

PBP: PPAR Binding Protein

PCN: Pregnenolone 16α-carbonitrile

PDGF: Platelet Derived Growth Factor

PEPCK: Phosphoenolpyruvate Carboxykinase

PGC-1α: Peroxisome Proliferator Activated Receptor Gamma Co-activator 1 Alpha

PI3K: Phosphatidylinositol 3-Kinase

PKA: Cyclic-AMP-dependent Protein Kinase

PKB: Protein Kinase B

PKC: Protein Kinase C

PMA: Phorbol Myristate Acetate

PPAR: Perioxisome Proliferator Activated Receptor

PR: Progesterone Receptor

PXR: Pregnane X Receptor

PXR-KO: Pregnane X Receptor Knockout

RAR: Retinoic Acid Receptor

RIF: Rifampicin

ROS: Reactive Oxygen Species

RXR: Retinoid X Receptor

SCD1: Stearoyl-CoA Desaturase 1

SHP: Small Heterodimeric Partner

SMRT: Silencing-mediator for Retinoid and Thyroid Hormone Receptors

SNP: Single Nucleotide Polymorphism

SPA: Scintillation Proximity Assay

SR-B1: Scavenger Receptor Class B Type 1

SRC: Steroid Receptor Co-activator

SREBP: Sterol Regulatory Element Binding Protein

SRM: Selective Receptor Modulator

SUG-1: Suppressor for Gal-1

SULT: Sulfotransferase

TAT: Tyrosine Amino Transferase

tBHQ: Tertiary Butylated Hydroquinone

TNF4α: Tumor Necrosis Factor 4 Alpha

TORC2: Transducer of Regulated CREB Activity 2

UGT: UDP Glucuronosyltransferase

VDR: Vitamin D Receptor

XREM: Xenobiotic Responsive Enhancer Module

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Chapter 1: Cell Signaling and Nuclear Receptors: New Opportunities for Molecular Pharmaceuticals in Liver Disease

1.1 Introduction

1.1.1 General NR Structure and Function

NRs are one of the largest groups of transcription factors with 48 members in the human genome that regulate diverse biological processes including metabolism, homeostasis, development and reproduction [1]. The activity of many NRs is controlled by the binding of small lipophilic molecules such as hormones, fatty acids, bile acids and oxysterols, and xenobiotics.

All members of the NR superfamily share several conserved structural domains that are essential for receptor function [2]. The C-terminal region encompasses the ligand-binding domain (LBD) and includes a region termed activation function 2 (AF-2), which is an important site for co-activator protein-binding. Binding of ligand induces a conformational change that creates a new surface for the recruitment of co-activator proteins in the AF-2 region [3]. The LBD is connected to a DNA binding domain (DBD) by a hinge region (H) that contains a nuclear localization signal. The DBD is highly conserved and contains two alpha helices and two zinc fingers that are involved in the specificity of response-element-recognition and in receptor dimerization. Most liver-enriched NRs are active as dimers, functioning either as homodimers, or as heterodimers with retinoid x receptor (RXR) [4]. Vertebrate RXR includes at least three distinct genes (RXRα, RXRβ, and

RXRγ) that give rise to a large number of protein products through differential promoter usage and alternative splicing.

The N-terminal region of NRs is highly variable in sequence and length, but all contain a region termed activation function 1 (AF-1) that acts independently of ligand [5]. The AF-1 domain contains many consensus phosphorylation sites and is therefore, the target of multiple kinases. Although most of the phosphorylation sites identified in NRs are located in the N-terminal domain, many receptors have at least one phosphorylation site in the H region, and there are limited reports of sites located in the LBD and DBD. In addition, there are likely many yet to be identified phosphorylation sites in NRs.

1.1.2 Intracellular Localization

Most NRs are constitutively localized in the nucleus, however, the major proportion of steroid receptors and a few other exceptional receptors may be located in the cytoplasm in the absence of ligand. Nuclear localization of NRs is mainly regulated by protein-protein interactions such as dimerization with RXRs or co-regulator proteins [6]. In the cytoplasm, NRs are bound to heat shock proteins and this association prevents receptor transportation through the nuclear pores and thus sequesters NRs from binding to DNA [7]. In the nucleus, ligand-mediated activation of NRs causes redistribution of the receptor to chromatin. Recent evidence, which will be discussed in more detail, has suggested that nuclear localization of some NRs is a cell signaling- and phosphorylation-dependent event.

1.1.3 Co-regulator Proteins

The full activity of NRs depends on a large number of co-regulator proteins that do not bind to DNA directly, but have a pronounced effect on the outcome of gene expression [3]. In general, non-liganded NRs form a complex with co-repressor proteins that inhibit transcriptional activity, often through the recruitment of other cofactor proteins that contain histone deacetylase (HDAC) activity. HDACs alter chromatin structure by promoting chromatin compaction, thus rendering enhancer regions of genes less accessible to the necessary basal transcriptional machinery. Activation of NRs by ligand-binding or through phosphorylation induces a conformational change which results in the dissociation of the co-repressor multiprotein complexes and subsequent recruitment of co-activator protein complexes that enhance the rate of gene transcription, often through the recruitment of multiprotein complexes containing histone acetyltransferase (HAT) activity. Co-regulator proteins thus provide a second level of specificity in the modulation of gene expression by NRs. Most NR-co-activator proteins identified to date preferentially interact with NRs through the C-terminal AF-2 domain via an -LXXLL- motif, which constitutes a prototypical NR-interaction motif. However, in contrast to most co-activator proteins, the peroxisome proliferator activated receptor gamma co-activator 1 alpha (PGC-1α) interacts not only with the AF-2 region of NRs, but also with the H region of the selected liver-enriched NRs [8]. In addition to NRs, it has also been shown that the intrinsic and recruited enzymatic activities of several NR-associated co-factor proteins are regulated by phosphorylation in a dynamic manner in response

to specific signal transduction pathways, and this will be discussed later in this chapter in more detail.

1.2 Liver-Enriched NRs as Targets of Signal Transduction Pathways

Figure 1-1 outlines the interface between NRs and the signal transduction pathways discussed in this chapter. While tyrosine phosphorylation of selected NRs has been observed, the functional significance of this type of phosphorylation is unknown. The majority of amino acid residues identified as being regulated-phosphorylation sites are serine and threonine residues. Many of these sites lay within the N-terminal AF-1 region of NRs and correspond to consensus sites for proline-dependent kinases such as cyclin-dependent kinases [9, 10] and mitogen-activated protein kinases (MAPKs) [11]. For some NRs, such as progesterone receptor (PR), which contains at least 13 sites, phosphorylation of the N-terminus is quite complex. However, other NRs such as peroxisome proliferator activated receptors (PPARs) contain only one or two phosphorylation sites in the N-terminus [12].

NRs can be phosphorylated constitutively in the absence of ligand, or in response to ligand-mediated activation. Other NRs can be phosphorylated independently of ligand in response to cellular signaling events by MAPKs. For example, growth factors, stress, cytokines, and other signals activate several serine kinase cascade pathways that activate different MAP kinases, including extracellular signal-regulated kinase (ERK), Jun-N-terminal kinase (JNK), or p38 MAPK which can enter the nucleus and phosphorylate NRs. The N-terminal domains of PR [13,

14], estrogen receptor alpha (ERα) [15, 16], estrogen receptor beta (ERβ) [17, 18], androgen receptor (AR) [19], PPARs [20, 21], and retinoic acid receptor gamma (RARγ) [22, 23] have all been reported to be substrates of ERK or p38 MAPK, and that of RXRα is phosphorylated by JNK. In addition to MAPK sites, the N-terminus of many NRs also contains consensus sites for Akt kinase, or protein kinase B, a kinase critical for cell survival and proliferation [24, 25]. Activated Akt negatively regulates downstream MAP kinases [26], and upon nuclear translocation phosphorylates specific NRs including ERα [27] and AR [28].

In addition to the N-terminal domain, the LBD and DBD are also targets for protein kinases. Phosphorylation of the LBD can involve the same proline dependent kinases. For example, serine residues contained within the LBD of RXRs are targeted by the stress activated protein kinase- JNK [29, 30]. Phosphorylation by other kinases such as tyrosine kinases for ERα [31, 32] and RXRα [30] or cyclic-AMP-dependent protein kinase (PKA) for RAR [33] is also common. Evidence for phosphorylation of the DBD involves either PKA for ERα [34] or PKC for RARα [35] and vitamin D receptor (VDR) [36, 37].

While signal transduction pathways and phosphorylation regulate most, if not all NRs in multiple tissue types, this chapter will focus on the regulation of non-steroid NRs expressed in a liver-enriched manner.

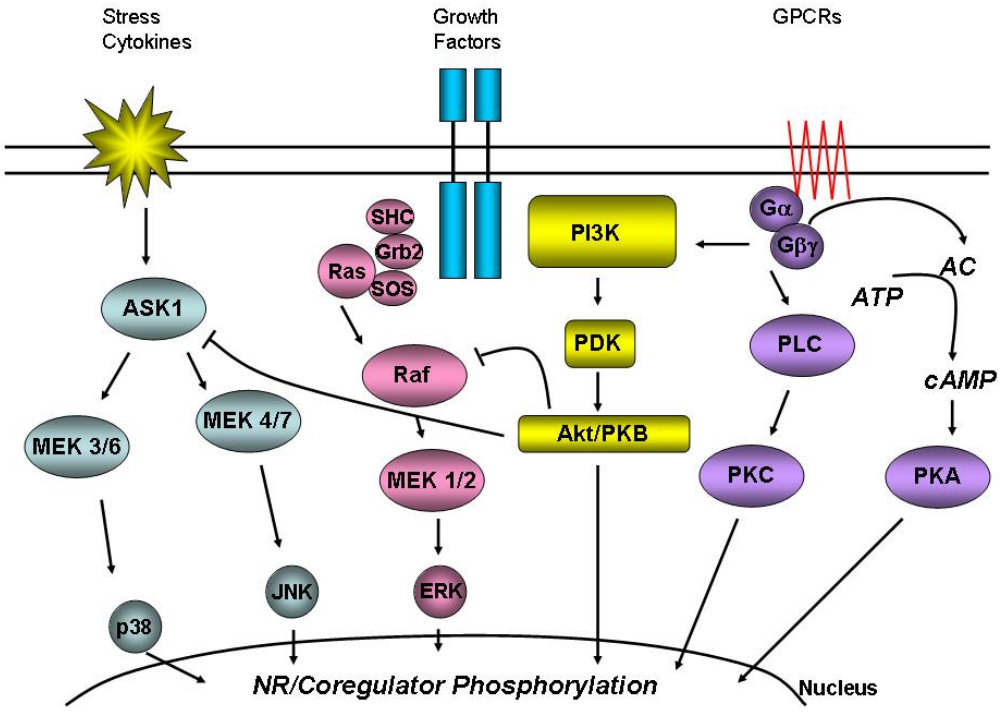

Figure 1-1. Activation of signaling pathways modulates nuclear receptor transcriptional activity. Activation of MAPK signaling cascades (p38, JNK, and ERK), FGF signaling, and G protein-coupled receptor (GPCR) signaling results in phosphorylation-dependent modulation of NR activity. Signaling pathways and phosphorylation events affect nuclear receptors or nuclear receptor cofactors through the modulation of protein–protein interactions, subcellular localization, DNA-binding, protein stability, and transactivation capacity. The interface between signal transduction pathways and NRs is critical in the responsiveness of the system to environmental and physiological stimuli.

1.2.1 NR2A1, HNF-4α

Hepatic nuclear factor-4α (HNF-4α) is a NR expressed mainly in liver, intestine, and kidney and is critical for development and liver specific gene expression [38]. HNF-4α has been implicated in the regulation of many genes in liver such as *Cyp7a1*[39], the constitutive androstane receptor (*CAR*) [40], and genes involved in glucose transport and glycolysis [41]. Although typically thought of as an orphan receptor, HNF-4α has been shown to be activated by fatty acyl-CoA thioesters [42]. In addition, the transcriptional activity of HNF-4α is regulated by phosphorylation of serine, threonine, and tyrosine residues. Phosphorylation of HNF-4α is required for DNA binding and appropriate sub-nuclear localization. Violett *et al.* indentified a PKA consensus phosphorylation site in the DBD of HNF-4α and report that HNF-4α is directly phosphorylated by PKA. PKA-mediated phosphorylation of wild-type HNF-4α strongly repressed the binding affinity and transcriptional activity of the receptor based on gel-shift assays and reporter gene analysis [43]. On the other hand, phosphorylation of specific serine and threonine residues in HNF-4α alters its tertiary structure, which increases the affinity and specificity of DNA-binding in COS-7 cells [44]. In addition to alterations in DNA-binding, tyrosine phosphorylation is required for appropriate sub-nuclear localization and transactivation activity of HNF-4α as evidenced by immunofluorescence, electron microscopy, and reporter gene assay using genistein treatment to inhibit tyrosine phosphorylation [45].

It has also been recently shown that p38 kinase phosphorylates HNF-4α at S158 increasing its interaction with co-activator PC4, DNA-binding, and transactivation activity in the presence of interleukin (IL)-1β and hydrogen peroxide [46]. In addition, inhibition of p38 kinase activity diminishes HNF-4α nuclear protein levels and its phosphorylation, rendering a less stable protein. Induction of p38 kinase by insulin results in an increase of HNF-4α protein and *Cyp7a1* gene expression in primary rat hepatocytes, thus providing a functional link between HNF-4α phosphorylation and bile acid synthesis [47]. Since HNF-4α has been shown to activate multiple genes and interacts with multiple transcription factors and co-regulators in liver such as chicken ovalbumin upstream promoter transcription factors (COUP-TFs), steroid receptor co-activator (SRC) proteins, CREB binding protein (CBP), p300, and PGC-1α [40, 48, 49], further study is required to determine the mechanism by which phosphorylation of HNF-4α modulates protein-protein interactions and differential gene expression.

1.2.2 The NR1C Subfamily- PPARs

The three peroxisome proliferators activated receptor (PPAR) isotypes, PPAR α, β, and γ, form a subfamily of NRs that are mainly involved in lipid and glucose homeostasis, control of inflammation and wound healing, and regulation of food intake and body weight [50, 51]. PPARα is expressed in metabolically active tissues including liver, kidney, heart, skeletal muscle and brown fat. PPARγ is expressed to a high extent in adipose tissue with lower amounts present in kidney, liver, and skeletal and smooth muscle [52]. Fatty acids and fatty acid derivatives are

endogenous ligands for PPARs and induce PPAR-dependent gene activation. In addition, PPARs are very important therapeutic targets for the treatment of hyperlipidemia and type-2-diabetes. The hypolipidemic fibrates were the first known synthetic ligands of PPARα, while thiazolidinediones are the best characterized PPARγ ligands used in the treatment of type-2-diabetes [53]. Therefore, understanding the mechanisms that regulate PPAR activity is crucial for effective therapeutic treatment of metabolic diseases.

Insulin treatment enhances PPARα activity via phosphorylation of S12 and S21 by p42/p44 MAP kinase, but represses PPARγ activity via phosphorylation of S112 [20, 21]. Furthermore, phosphorylation of the N-terminal domain of PPARγ has been shown to decrease PPARγ activity. For example, platelet-derived growth factor (PDGF) treatment decreases PPARγ transcriptional activity in reporter gene assays, and *in vivo* labeling experiments demonstrated that PPARγ undergoes epidermal growth factor (EGF)-stimulated MAPK-dependent phosphorylation at S82 [54]. Further studies indicate that PPARγ activity is decreased through phosphorylation of the N-terminus at S84 by ERK2 and JNK via tumor necrosis factor (TNF)4α and EGF stimulation [55]. In contrast, another study reports an increase in transactivation of PPARγ via insulin-stimulated ERK2 phosphorylation in CHO cells [56]. Differential modulation of PPAR activity by phosphorylation is likely due to the relationships between specific kinases, serine residues, ligands, and receptor isoforms in specific cell types. For example, ERK2 and JNK, but not p38 MAP kinase can

phosphorylate PPARγ on S84 [55], while PPARα is a substrate for both ERK2 and p38 MAP kinase in a ligand-dependent manner [57]. In addition, phosphorylation of PPARα by PKA in transient reporter gene assays was shown to have different effects depending on which promoter was used experimentally [58].

There are multiple mechanisms by which phosphorylation of PPARs modulates their activity. Transfection studies have suggested that phosphorylation of the N-terminus induces the dissociation of co-repressor proteins such as nuclear receptor co-repressor (NCoR) from PPARα [21]. In a similar fashion, phosphorylation of the N-terminus of PPARα increases co-activation by PGC-1α [57]. Phosphorylation can also enhance DNA-binding of PPARs, as is the case with PPARα phosphorylation by PKA [58]. PPARs may also be modulated through kinase cascades that up-regulate their own expression as shown by the PKC-dependent upregulation of PPARα gene expression [59].

MAPKs, PKA, and PKC are three kinase families that have been implicated in the phosphorylation of PPARα and γ. Activation of these signaling pathways and phosphorylation of PPARs could affect the endogenous and therapeutic function of PPARs. For example stress or fasting may activate PKA signaling which phosphorylates PPARα, enhances its activity through the recruitment of PGC-1α, and may affect the function of PPARα as a drug target.

1.2.3 NR1I2, PXR

Pregnane x receptor (PXR) is a master-regulator of xenobiotic-inducible cytochrome-p450 (CYP) gene expression in liver. The CYPs identified as PXR

target-genes encode enzymes responsible for the oxidative metabolism of over 60% of clinically prescribed drugs. In addition, several studies have shown that PXR regulates other genes involved in the metabolism of xenobiotic and endobiotic compounds such as glutathione S-transferases (GSTs), sulfotransferases (SULTs), and UDP-glucuronosyltransferases (UGTs) [60-63]. PXR also regulates the expression of the drug-transporter genes organic anion transporting polypeptide 2 (Oatp2), multi-drug-resistance 1 (Mdr1), multi-drug resistance-associated protein 2 (Mrp2), and multi-drug resistance-associated protein 3 (Mrp3) [64-66]. PXR is a promiscuous receptor activated by a wide variety of compounds including synthetic and endogenous steroids, bile acids, and a variety of drugs and natural compounds [67]. In this manner, the modulation of PXR activity by ligands and/or signaling pathways represents the basis for an important class of drug-drug interactions.

Drug-inducible CYP gene expression is known to be responsive to cytokine, PKC, and PKA signaling pathways, however the exact mechanism by which these pathways intersect with PXR is unknown. For example, a significant reduction in the hepatic expression of *Mdr1* and *Mrp3* genes were seen in endotoxin treated mice. Similarly, IL-6-treated mice displayed a 40-70% reduction in the mRNA levels of all Mdr isoforms [68]. Inflammatory cytokines inhibit the inducible expression of Oatp2 during intrahepatic cholestasis [69]. It has also been shown using primanry cultures of human hepatocytes that IL-6 markedly decreases the expression of PXR and its close cousin CAR. IL-6 also decreases both rifampicin- and phenobarbital-mediated induction of CYP3A and CYP2B gene expression [70].

Recent evidence has demonstrated crosstalk between PXR and nuclear factor kappaB (NF-κB) signaling pathways. NF-κB activation by lipopolysaccharide (LPS) and TNF4α inhibited PXR association with the CYP3A promoter by disrupting the interaction between the PXR-RXR protein complex [71]. In addition, PXR activation inhibited the activity of NF-κB and the expression of its target genes. This inhibition was shown to be PXR-dependent and was potentiated by PXR ligands *in vitro* and *in vivo* [72]. PXR activation has also been shown to alleviate the symptoms of inflammatory bowel disease. Studies using inflammatory bowel disease and PXR knockout mouse models have shown that PXR agonist treatment decreased the expression of NF-κB target gene expression in a PXR-dependent manner [73].

In addition to cytokine signaling, CYP3A gene expression is also modulated by PKA and PKC signaling pathways. Co-treatment of primary cultures of rat hepatocytes with phenobarbital and cyclic AMP analogs and PKA activators clearly results in cyclic AMP-associated inhibition of CYP gene expression [74]. However, treatment with the andenyl cyclase activator forskolin and its non-PKA-activating analog 1,9 dideoxyforskolin both resulted in the stimulation of CYP3A gene expression [75]. Ding and Staudinger have shown that both forskolin and 1,9 dideoxyforskolin induce CYP3A expression in primary mouse hepatocytes by functioning as PXR agonists. In addition, activation of PKA signaling potentiated PXR-mediated induction of CYP3A expression and increased the strength of PXR-co-activator protein interactions in mammalian 2-hybrid reporter gene assays. Further kinase assays show that PXR can be a substrate for PKA *in vitro*, suggesting a

potential mechanism for PKA-mediated modulation of CYP3A gene expression [76]. It is also of interest that while PKA activation potentiates the expression of CYP3A in mouse hepatocytes, it is a repressive signal in both human and rat hepatocyte cultures. This suggests a species-specific effect for the modulation of CYP3A by PKA signaling. Differential phosphorylation may be a possible factor the species-specific responses to PKA signaling. In addition, activation of PKC signaling dramatically represses PXR activity in reporter gene assays and in hepatocytes by increasing the strength of interaction between PXR and the co-repressor NCoR, and by abolishing the ligand-dependent interaction between PXR and co-activator SRC-1 [77].

1.2.4 NR1I3, CAR

Similar to PXR, the NR superfamily member CAR was first classified as a xenobiotic-sensing transcription factor that regulates numerous hepatic genes in response to a large group of xenobiotics and endobiotics. CAR was originally found to regulate the transcription of genes encoding the CYP2B subfamily [78]. In addition to CYP2B, CAR also regulates the expression of multiple drug and hormone metabolizing enzymes and transporter proteins such as CYP3A, CYP2C, GSTs, SULTs, UGTs, Oatp2, Mrp2 and Mrp3 [79]. Interestingly, treatment of wild type and CAR knockout mice with phenobarbital, the prototypical CAR activator, both induces and represses certain hepatic genes in a CAR-dependent manner suggesting that CAR has diverse roles as both a positive and negative regulator of hepatic gene expression in response to Phenobarbital (PB) [80]. As the function of CAR has expanded, so has interest in the deciphering the molecular mechanism of its activation by drugs.

It is well documented that the PB-mediated induction of CYP2B genes in cultured hepatocytes is responsive to several serine/threonine protein kinases and phosphatases. However, the mechanism by which these signaling pathways interact with CAR remains poorly understood. For example, activation of PKA signaling negatively impacts the induction of CYP2B expression in primary cultures of rat hepatocytes [74], and co-treatment with phosphatase inhibitors further potentiates the repressive effects of PKA signaling [81]. Serine/threonine-specific protein phosphatases PP1 and PP2A have a positive role in the induction of CYP2B [82]. Pustylnyak *et al.* further reports that rats treated with inhibitors of Ca(2+)/calmodulin-dependent kinase exhibited increased gene expression of both CAR and CYP2B, while rats treated with the protein phosphatase PP1 and PP2A inhibitor okadaic acid exhibited the opposite effect [83].

In the absence of a ligand or activator, CAR is sequestered in the cytoplasm where it forms a complex with heat shock protein 90 (Hsp90) and cytoplasmic-CAR-retention-protein (CCRP). In response to PB the complex recruits protein phosphatase 2A before translocation of CAR to the nucleus. The protein phosphatase inhibitor okadaic acid represses PB-induced nuclear translocation of CAR [84]. In addition, de-phosphorylation of S202 in mouse CAR is required for its nuclear translocation [85]. The signaling pathway involved in the phosphorylation of S202 remains unknown. Unlike most NRs, CAR translocates to the nucleus without directly binding PB [86]. Taken together, these data indicate that the phosphorylation

status of CAR is intimately involved in its cytoplasmic retention and nuclear translocation.

Recent evidence shows that EGF represses PB-mediated activation of CAR-dependent transcription [87], and that the MEK inhibitor U0126 increases the PB-mediated induction of CYP2B in primary rat heptocytes [88]. In addition, hepatocyte growth factor (HGF) treatment represses induction of cyp2b10 by PB in primary mouse hepatocytes. HGF treatment increased the phosphorylation of ERK1/2, thus decreasing the nuclear translocation of CAR [89], however, the exact mechanism by which this occurs is unknown. In addition to MEK/ERK signaling, AMP-activated protein kinase (AMPK) has been suggested to activate CAR in hepatocytes [90]. Additional studies using AMPK knockout mice demonstrate that that although AMPK does not regulate the PB-induced translocation of CAR, it may be involved in the activation of CAR in the nucleus [91]. Shindo et al. reported that activation of AMPK resulted in nuclear accumulation of CAR but was not sufficient to induce CYP2B gene expression [92]. Additional studies suggest that PB targets LKB1 kinase for the activation of AMPK [93], adding a proximal target to the elusive sequence of events by which PB activates transcription of CYP2B. While AMPK appears to be an activating signal for PB-mediated induction of CYP2B, MEK/ERK seems to be repressive. Further study into the signal-related mechanisms of CAR activation is required to determine the effect that these pathways might have on the phosphorylation status of CAR or CAR-interacting proteins.

1.2.5 NR1H3, LXR

Liver X receptors (LXRα and LXRβ) have emerged as important regulators of cholesterol metabolism and transport, lipid metabolism, glucose homeostasis and inflammation [94]. LXRα is primary expressed in liver, macrophages, and adipose tissue, while LXRβ is more ubiquitously expressed [67]. LXR-activating ligands include several oxysterols and 6α-hydroxy bile acids [95, 96]. Since the discovery of LXRs, multiple LXR-target genes that are involved in cholesterol and lipid homeostasis have been identified. These include *CYP7A1*, the rate limiting enzyme in the classical pathway of bile acid synthesis, ATP-binding cassette transporters, lipoproteins such as apolipoprotein E, lipoprotein lipase, and lipogenic proteins such as sterol response element binding protein-1C (SREBP-1C) and fatty acid synthase [97-100]. Although early reports emphasized the role of LXR in cholesterol homeostasis, recent studies suggest that LXR negatively regulates gluconeogenesis [101] and inflammatory responses [102, 103].

LXR has been shown to exist as a phosphoprotein in HEK293 cells. Mutational analysis and metabolic labeling indicate that LXR is constitutively phosphorylated at S198 in the hinge region of the receptor at a MAPK consensus site [104]. However, the biological significance of this phosphorylation event has yet to be elucidated.

Early studies demonstrate that PKA/PKC modulators such as prostaglandin E2, phorbol esters, 8-bromo-cyclic AMP, and forskolin enhanced the induction of reporter genes by LXR ligands [105]. These experiments suggest that trans-activation

by ligand-activated LXR may be further modulated through kinase signaling. PKA can directly phosphorylate LXR, and has been reported to both increase and decrease trans-activation depending on the experimental conditions [106-108]. In primary cultures of rat hepatocytes, activation of PKA repressed LXR-mediated *SREBP-1C* gene expression. Direct phosphorylation of LXR by PKA *in vitro* and *in vivo* at two PKA consensus sites (S195, S196 and S290, S291) located in the LBD was required for trans-repression. PKA-mediated phosphorylation of LXR impaired DNA-binding through the disruption of LXR/RXR dimerization, and decreased transcriptional activity by inhibiting the recruitment of the co-activator protein- SRC-1, and enhancing the recruitment of co-repressor protein- NCoR [108]. On the other hand, Tamura et al. have demonstrated that PKA signaling can increase LXR trans-activation in reporter gene assays conducted in renal As4.1 mouse cell lines [106].

In addition to inducing genes involved in cholesterol and glucose homeostasis, LXR reciprocally represses a set of inflammatory genes including inducible-nitric oxide synthase, cyclooxygenase-2, IL-6, and matrix metaloproteinase-9 after bacterial, LPS, TNFα or IL-1β stimulation [102, 103]. Importantly, LXR agonists reduce inflammation *in vivo*. The mechanism by which LXR represses inflammatory genes is not well understood. No LXR response elements have been identified on the promoters of the repressed genes. In addition to possible competition for co-regulator proteins, recent evidence suggests that inhibition of the NF-κB pathway is involved likely through trans-repression of NF-kB in the nucleus [109]. In a recent study of trans-repression of the iNOS promoter, SUMOylation of PPARγ was identified as a

mechanism of repression [110]. In a similar manner, SUMOylation of LXR has been shown to negatively regulate a subset of pro-inflammatory genes by preventing the removal of NCoR co-repressor complexes from the promoter [111]. It remains to be determined the extent to which post-translational modification of LXR is involved in LXR-mediated trans-repression of inflammatory genes.

1.2.6 NR5A2, LRH-1

The orphan NR liver receptor homolog 1 (LRH-1) functions to regulate the expression of a number of genes involved in bile acid homeostasis and other liver functions. Unlike the majority of NRs that function as dimers, LRH-1 binds as a monomer to an extended NR half site in the promoter of its target genes [112]. LRH-1 is an important regulator of *CYP7A1* gene expression [113, 114]. LRH-1 also regulates the expression of other genes involved in cholesterol and bile acid homeostasis including *CYP8B1*, *Mrp3*, cholesterol ester transfer protein, apical sodium-dependent bile acid transporter, and apolipoprotein A1 [60, 115-120].

No ligands for LRH-1 have yet been identified, and the mechanisms that modulate its activity are still unclear. Lee *et al.* have shown that treatment with the phorbol ester PMA increases LRH-1 activation in Hela cells. The ERK1/2 inhibitor U0126 blocks this response. Mutation analysis confirms that phosphorylation of LRH-1 at S238 and S243 in the H domain stimulates LRH-1 transactivation [121]. In contrast, activation of JNK pathways is associated with inhibitory effects on the LRH-1 target *CYP7A1*; however the role of LRH-1 in this pathway is unclear [122, 123].

Treatment of HepG2 cells with the inflammatory cytokine- TNFα, a potent activator of the JNK pathway, increased the expression of LRH-1 and MRP3, and also increased LRH-1-binding to the MRP3 promoter [115]. Krylova et al., have suggested that phosphatidylinositols, major intracellular signaling molecules, bind to LRH-1, linking phospholipid signaling and gene expression [124]. However the functional role of phosphatidylinositols as modulators of LRH-1 function remains unknown.

1.2.7 NR1H4, FXR

Farnesoid x receptor (FXR) is a NR expressed in liver, intestine, kidney and adipose tissue. FXR has emerged as a key player involved in the maintenance of cholesterol and bile acid homeostasis through its regulation of the expression of genes involved in the synthesis, uptake, and excretion of bile acids [125]. An important breakthrough in the FXR field was the discovery that FXR is directly activated by several bile acids including chenodeoxycholic acid, lithocholic acid, and deoxycholic acid [126-128]. Studies with FXR knockout mice revealed that a number of genes involved in cholesterol homeostasis are also regulated by FXR including *Cyp7a1*, *Cyp8b1*, intestinal bile acid binding protein, canalicular bile salt excretory pump, phospholipid transfer protein, and the hepatic basolateral transporter- Na (+)-taurocholate-cotransporting polypeptide [129]. Additional studies reveal that FXR induces expression of the NR superfamily member- small heterodimeric partner (SHP). Increased SHP then represses *Cyp7a1* transcription by inhibiting the activity of LRH-1, which is a positive regulator of the *Cyp7a1* promoter [113].

Although there is no evidence at this time for direct phosphorylation of FXR, the expression and activity of FXR are modulated by signal transduction pathways. For example, FXR is thought to modulate insulin signaling. FXR expression is reduced in streptozotocin-induced diabetic rat models, and administration of insulin restores FXR mRNA to normal levels [130]. Additional support for this concept comes from the observation that glucose reduces FXR expression in the liver [130], while activation of FXR by the synthetic agonist GW4064 reduces plasma glucose levels in mice [131]. Furthermore, loss of FXR disrupts normal glucose homeostasis and leads to the development of insulin resistance in FXR knockout mice [132-134]. FXR may also play a role in regulating glucose and lipid metabolism during alterations in nutritional status. A recent study reports that FXR expression is induced in mouse liver in response to fasting, a condition during which PKA signaling is enhanced [135].

Bile acids that are FXR agonists have been shown to activate multiple signal transduction pathways. Treatment with taurocholic acid results in activation of the JNK pathway [136], and deoxycholic acid treatment activates the Raf-1/MEK/ERK signaling cascade in primary rat hepatocytes [137]. In addition, treatment of HepG2 cells with bile acids results in the activation of PKC, and treatment with PKC inhibitors reduces the bile acid-mediated repression of *Cyp7a1* gene expression [138]. While there is evidence for multiple bile acid-responsive pathways, future research goals include elucidation of the effects that kinase activation has on the phosphorylation status and functional activity of FXR.

1.2.8 NR0B2, SHP

The NR SHP is an atypical orphan member of the NR superfamily in that it lacks the conserved DBD. SHP was isolated in a yeast 2-hybrid screen based on its ability to dimerize with other NRs [139]. It is expressed mainly in liver, small intestine, spleen, heart and pancreas [140]. SHP interacts with a variety of NRs in liver including PPARα [141], LRH-1 [113, 142], LXR [143], and HNF4α [144]. SHP acts as a direct transcriptional repressor and inhibits the activity of most NRs with which it interacts [145]. Two notable exceptions are that SHP enhances the transcriptional activation of PPARα [146], and PPARγ [147] under certain conditions.

It has been shown that SHP expression is regulated by the JNK pathway. Gupta *et al.* provide evidence that bile acids rapidly down-regulate *CYP7A1* transcription via activation of the JNK pathway, and that *SHP* is a direct target-gene of activated c-Jun [136]. Over-expression of c-Jun resulted in increased SHP promoter activity, whereas mutation of the c-Jun response element in the *SHP* promoter abolished activation and induction of reporter gene expression under the control of the *SHP* promoter. This study provides an alternative mechanism for bile acid-mediated induction of SHP expression that is independent of FXR.

1.2.9 NRs and FGFs

Recent evidence has uncovered several novel NR-dependent mechanisms involving fibroblast growth factors (FGFs). This recent thrust of research has created a new paradigm that particular FGFs function as metabolic hormones and act through

yet to be described signal transduction cascades to elicit specific physiological responses. FGFs function in processes such as development and wound healing. However, three members of the FGF family, FGF19 (FGF15 in mouse), FGF21, and FGF23 have recently emerged as novel metabolic hormones. The expression of FGF23 plays a role in calcium and phosphate homeostasis and is regulated by VDR, which is expressed mainly in bone, kidney and intestine and therefore will not be discussed in this chapter [148, 149].

As mentioned earlier in this chapter, activation of FXR by bile acids down-regulates *Cyp7a1* gene expression in mice through an indirect mechanism involving the induction and activation of the negative regulator- SHP. Recently, an additional FXR-dependent mechanism involving FGF19 has been described. FGF19 binds to its cell surface receptor, FGF-receptor (FGFR)4, and increases JNK-dependent signaling [150]. In primary cultures of human hepatocytes, FXR activation induces expression of FGF19. FGF19 then modulates bile acid biosynthesis by reducing the expression of CYP7A1 through a JNK-dependent pathway without affecting SHP expression [151]. It has also been shown that over-expression of FGF19, using either a transgenic approach or with chronic FGF19 treatment, improves insulin sensitivity and glucose homeostasis in diet-induced obese mice, in part through increased metabolic rate and fatty acid oxidation [152, 153]. Conversely, activation of the JNK signaling pathway in the liver has been shown to increase insulin resistance [154], however there is currently no explanation for this discrepancy.

FGFR4 deficient mice exhibit reduced JNK activity, an increased bile acid pool, and enhanced expression of CYP7A1 [155]. On the other hand, transgenic mice expressing constitutively active FGFR4 exhibit increased JNK activity, a reduced bile acid pool, and reduced expression of CYP7A1 [156]. The expression of FGF15, the mouse ortholog of FGF19, is induced by FXR activation in the small intestine, but not in the liver. FGF15 expression then represses CYP7A1 in liver through a mechanism that involves FGFR4 and SHP [157]. In addition, mice lacking FGF15 have increased hepatic Cyp7a1 expression and activity corresponding to increased bile acid excretion [157]. Taken together, these studies define FGF19 in humans and FGF15 in mice as pivotal components of a novel signaling pathway that cooperates with FXR and SHP to maintain bile acid homeostasis.

PPARα, a fatty acid-activated NR, regulates the utilization of fat during the starvation response. Recently, a PPARα-dependent role for FGF 21 in the adaptive response to starvation has been described. FGF21 has been observed to have a variety of beneficial effects on metabolic parameters. Treatment of obese and leptin-deficient mice with FGF21 decreases serum glucose and triglyceride concentrations, and increases insulin sensitivity and glucose clearance. Moreover, mice that over-express FGF21 are resistant to diet induced obesity [158]. Similar results were observed in FGF21 treatment of diabetic rhesus monkeys [159]. While theses studies show that the administration of FGF21 has important metabolic effects, recent studies have provided insight to the physiological role of FGF21. Inagaki *et al.* and Badman *et al*., show that FGF21 expression in the liver of fasted mice is induced following

activation of PPARα [160, 161]. Adenoviral knockdown of endogenous FGF21 resulted in fatty liver, increased serum triglyceride levels, and decreased serum ketone levels in mice fed a low carbohydrate, high fat diet [160]. This was associated with the decreased expression of fatty acid oxidizing enzymes and key enzymes in ketone body production that are known PPARα-target genes [160]. In transgenic mice over-expressing FGF21, ketogenesis and ketone body concentration in serum was increased several fold. Interestingly, recombinant FGF21 treatment rescued defective ketone body production in PPARα knockout mice [161]. Over-expression of FGF21 in mice also produces decreased body temperature and locomotor activity during fasting and increased lipolysis in white adipose tissue [161]. These studies make it evident that FGF21 signaling collaborates with PPARα, and together they function in liver as master regulators of energy balance. The precise molecular mechanisms that are downstream of these FGF signaling pathways in liver remain to be elucidated.

1.3 Co-regulator Proteins as Targets of Signal Transduction Pathways

Interaction of NRs with co-regulator proteins provides a second level of regulation in target gene activation. The association of co-regulator proteins with NRs is clearly controlled at the level of ligand binding. In addition, the activation of cell signaling events and/or protein kinases directly regulates the association of NRs with co-regulator proteins. Numerous examples cited above illustrate how phosphorylation of NRs can result in increased or decreased strength of interaction between the receptor and co-activator or co-repressor multiprotein complexes.

Realization that the specificity and activity of co-regulator proteins may also be regulated by signal transduction and phosphorylation is relatively new concept for which much less is known.

1.3.1 NCoR and SMRT Co-repressor Proteins

The most extensively characterized co-repressor proteins for NRs are NR co-repressor (NCoR) and silencing-mediator for retinoid and thyroid hormone receptors (SMRT) [162, 163]. NCoR and SMRT interact with and mediate the repression of overlapping sets of NRs. NCoR and SMRT do not have intrinsic enzymatic activity; instead they have conserved modular domains that interact with HDACs. These co-repressor proteins can bind to NRs at their conserved C-terminal receptor interacting domain in the presence or absence of ligand and are regulated by a variety of signal transduction pathways [164].

It had been previously observed that activation of tyrosine kinases negatively regulates the interaction between transcription factors and SMRT [165]. Further studies reveal that phosphorylation of SMRT in the C-terminal receptor-interaction domain by the MAP kinase-kinase MEK-1 and MEK-1 kinase (MEKK-1) inhibits the interaction between SMRT and NRs [166]. In addition, introduction of MEK-1 and MEKK-1 signaling into transfected cells leads to the redistribution of SMRT from the nucleus to the perinucleus or cytoplasm [166]. In contrast, phosphorylation of SMRT by casein kinase 2 (CK2) on S1492 stabilizes SMRT-NR interactions [167]. Therefore, different signaling pathways can modulate different transcriptional outcomes via SMRT phosphorylation.

In contrast to SMRT, NCoR is refractory to MEKK1 phosphorylation, does not release from NR partners and does not change its sub-cellular distribution in response to MEKK1 signaling [168]. These results indicate that the closely related SMRT and NCoR are regulated by distinct kinase signaling pathways. Although NCoR is fully refractory to MEKK-1 signaling, it is partially inhibited by EGF receptor signaling, indicating that NCoR may respond to an as yet undefined secondary pathway activated by EGF signaling [168]. Recent evidence suggests that this differential response may be determined by alternative mRNA splicing of SMRT and NCoR [169]. NCoR has been shown to be phosphorylated by Akt at S401, leading to the reversal of NCoR-mediated repression and nuclear export of NCoR. However, SMRT possesses and alanine residue at position 401 and is resistant to the actions of Akt [170].

Finally, since SMRT and NCoR exist in co-repressor multiprotein complexes, their activity may be affected by activation of signaling cascades that result in the phosphorylation of an HDAC, or other proteins in the complex. For example, phosphorylation of HDAC4 by ERK1 and ERK2 enhances its nuclear accumulation, whereas phosphorylation of HDAC1 and HDAC2 alters their interactions with co-repressor complexes [171-173]. IL-1β has been reported to inhibit NCoR through an indirect pathway resulting in the MEK kinase-1-mediated phosphorylation of the transforming growth factor-beta-activated kinase 1-binding protein 2 subunit present in a subset of NCoR-HDAC3 complexes, whereas SMRT is resistant to this pathway [174].

1.3.2 The p160/Steroid Receptor Co-activator Proteins

Steroid receptor co-activators (SRC) proteins are widely expressed and co-activate most NRs as well and many general transcription factors. The C-terminal region of SRC contains HAT activity, albeit relatively weak [175, 176]. Furthermore, SRCs recruit other co-activator proteins such as CBP, p300 and histone acetyltransferase p300/CBP-associated factor (pCAF) to a larger multiprotein complex that participates in chromatin remodeling [177]. There are three members of the SRC family, all of which contain conserved centrally located -LXXLL- motifs that are responsible for ligand-dependent interaction with NRs through the AF-2 domain [178].

Seven phosphorylation sites for SRC-1 and six for SRC-3 have been identified [179, 180]. All seven of the sites identified in SRC-1 contained consensus-phosphorylation sequences for serine/threonine-proline directed kinases, and two contained perfect consensus sequences for the MAPK family and are phosphorylated by ERK-2. Phosphorylation of SRCs can be induced by a variety of environmental stimuli including EGF, cyclic AMP, cytokines, and steroid hormones [179-183]. In addition, the phosphorylation of SRCs induced by these agents is required for optimal co-activator activity. For example, ERKs can phosphorylate SRC-2 at S736 and treatment of cells with EGF increases the transcriptional activity of GAL4-GRIP1 [184]. In addition, SRC-1 phosphorylation at S1185 and T1179 is induced by cyclic AMP, and phosphorylation at these sites enhances the ligand-dependent and -independent activity of PR [182]. PKA did not phosphorylate these sites in vitro, but

blockage of PKA activity in COS-1 cells prevents cyclic AMP mediated phosphorylation of these sites [182]. This phosphorylation event was also shown to be required for the interaction of SRC with pCAF or CBP [182]. In a similar manner, EGF-stimulated phosphorylation of SRC-3 by MAPK stimulates the recruitment of p300 and enhances ligand-dependent ER activity [181]. Phosphorylation of SRC-3 was shown to selectively affect the interactions with NRs, NF-κB, and CBP [180]. These data suggest that the phosphorylation of SRCs seems to be involved in the regulation of protein-protein interactions, however, it remains to be seen whether phosphorylation can affect other aspects of SRC-function.

All three SRCs contain both redundant and distinct functions which may be modulated by the signal transduction pathways that interact with SRCs and result in their phosphorylation. For example, SRC-3 contains several distinct patterns of phosphorylation. Phosphorylation of all six sites of SRC-3 is shown to be induced by estrogen and androgen hormones and is required for co-activation of estrogen and androgen receptors [180]. However, phosphorylation of only five of the six sites is induced by TNFα and is required for co-activation of NF-κB [180]. Further evidence shows that SRC-3, but not SRC-1 was co-purified in complex with IκB kinase, and consequently, phosphorylation of SRC-3, but not SRC-1 is enhanced in response to TNFα stimulation [183]. Thus, it appears that phosphorylation provides a molecular basis that determines the ability of SRCs to distinguish among various transcription factor families, and helps to provide specific responses to various upstream signaling pathways.

In addition to SRC, phosphorylation of other proteins in the co-activator complex can modulate transactivation potential of NRs. CBP and p300 can be phosphorylated *in vivo* and participates in cyclic AMP-regulated gene expression [185]. Kinase activities are also found to be associated with CBP and p300. Activation of cellular Ras with insulin treatment stimulated the recruitment of S6 kinase $pp90_{RSK}$ to CBP [186]. Binding of $pp90_{RSK}$ to CBP represses the transcription of cyclic AMP-responsive genes via the cyclic AMP response element binding protein (CREB) [186]. CBP also contains a signal-regulated transcriptional activation domain that is controlled by calcium/calmodulin-dependent protein kinase IV and by cyclic AMP [187]. Signal transduction pathways may also influence acetyltransferase activities and substrate preferences. An example of this is best illustrated with the POU homeodomain transcription factor Pit-1. Pit-1 function requires CBP, p300, and pCAF and is positively regulated by cyclic AMP and MAPK signal transduction pathways [188]. Interestingly, stimulation of Pit-1 activity by cyclic AMP requires the intrinsic HAT activity of CBP, whereas stimulation of Pit-1 activity by the MAP kinase pathway requires the HAT activity of pCAF [188]. It is thus plausible that activation of different signaling pathways could influence the group of co-activators that are required for NR mediated transactivation.

1.3.3 The PGC Family of Co-integrator Proteins

There are three members of the PPARγ co-activator (PGC) family, PGC-1α, PGC-1β, and the PGC-1 related co-activator- PRC. However, PGC-1α is the most extensively characterized member of the family. Like many protein cofactors, PGC-

1α co-activates multiple NRs. PGC-1α is selectively expressed mainly in skeletal muscle, cardiac muscle, white fat, and liver [189]. PGC-1α binds to NR-LBDs with high affinity and, similar to SRCs, contains a triplet -LXXLL- motif for binding to NRs through their AF-2 domains. PGC-1α does not have intrinsic HAT activity, and serves as a molecular scaffold that recruits additional factors such as CBP or p300 [190]. The physiological role of PGC-1α has been well characterized as a master regulator of energy homeostasis in fat, liver and muscle [191]. Specifically in liver, PGC1-α plays a prominent role in the regulation of genes involved in energy metabolism and glucose homeostasis. PGC-1α is induced in liver by fasting and up-regulates the expression of key genes that participate in gluconeogenesis [192], fatty acid oxidation [193], and bile acid synthesis [194].

PGC-1α interacts with a multitude of signaling pathways that affect both its expression and/or phosphorylation status. Agents that increase cyclic AMP signaling such glucagon, catecholamines, and glucocorticoids induce PGC-1α expression in liver [192]. This cyclic AMP/PKA-dependent induction of PGC-1α is mediated by phosphorylation and activation of the transcription factor CREB which directly regulates the *PGC-1α* promoter [195]. On the other hand, LKB1/AMPK signaling appears to regulate the repression PGC-1α gene expression. In LKB1 deficient liver, transducer of regulated CREB activity 2 (TORC2), a transcriptional co-activator of CREB, was de-phosphorylated and entered the nucleus, driving expression of PGC-1α [196].

In insulin stimulated skeletal muscle, PGC-1α gene expression is down-regulated by Akt-mediated phosphorylation and nuclear exclusion of the forkhead transcription factor FOXO1 [197]. In addition to insulin, obesity and saturated fatty acids decrease PGC-1α gene expression and function via p38 MAPK-dependent transcriptional pathways [198]. Moreover, palmitate, a common saturated fatty acid, reduces PGC-1α expression in skeletal muscle through a mechanism involving MAPK-ERK and NF-κB activation [199].

It is worth noting that the most prominent PGC-1α post-translational modification in terms of control of its activity and physiological output is acetylation [200, 201]. Methylation can also enhance PGC-1α activity [202]. However, this chapter will focus on the effect of phosphorylation of PGC-1α. Cytokines such as IL-1α, IL-1β, and TNFα have been shown to activate the transcriptional activity of PGC-1α in muscle through direct phosphorylation by p38 MAPK resulting in increased stability, half-life and activation of the PGC-1α protein [203]. p38 MAPK phosphorylates PGC-1α at three residues (T262, S265, and T298) that occur in a region previously shown to interact with NRs, however, it remains to be seen whether phosphorylation of PGC-1α affects NR docking. Further studies performed in primary hepatocytes confirm that PGC-1α phosphorylation by p38 MAPK is necessary for free fatty acid induced activation of *PEPCK*, a PGC-1α-target gene involved in gluconeogenesis [204]. The precise mechanism by which p38 MAPK-mediated phosphorylation of PGC-1α alters the amount and activity of PGC-1α will

likely provide important physiological, and perhaps therapeutic, insight. Kralli's group has also shown that activation of p38 MAPK leads to an increase in PGC-1α activity. They propose that a repressor binds to the PGC-1α –LXXLL- motif and that the interaction is terminated upon activation of p38 MAPK [205]. This suggests a model where the repressor and NRs compete to recruit PGC-1α to an inactive or active state, and that cellular signaling such as ligand or kinase signaling can shift the equilibrium between the two states.

In addition to p38 MAPK, two recent reports show that PGC-1α is phosphorylated by AMPK and Akt/PKB. AMPK activation in muscle increases the expression of genes required for glucose uptake, fatty acid oxidation, and mitochondrial biogenesis. Using primary muscle cells and PGC-1α knockout mice, Jager *et al.* demonstrated that the effect of AMPK mediated gene expression is dependent on PGC-1α function. Furthermore, AMPK phosphorylates PGC-1α at T177 and S583, which is required for PGC-1α dependent induction of the *PGC-1α* promoter [206]. In liver, the mechanism by which insulin regulates lipid synthesis and degradation are largely unknown. Insulin treatment, through protein kinase Akt2/protein kinase B (PKB) resulted in the phosphorylation and inhibition of PGC-1α [207]. Akt phosphorylates PGC-1α at S570 which prevents the recruitment of PGC-1α to its target promoters [207]. Repression of PGC-1α activity by phosphorylation impairs its ability to promote gluconeogenesis and fatty acid oxidation in liver. PGC-1α has an additional role in the regulation of this pathway.

PGC-1α co-activates PPARα in the expression of tribbles homolog TRB-3, a fasting inducible inhibitor of Akt/PKB [208]. This mechanism by which insulin signaling regulates PGC-1α activity could provide insight into alternative drug targets for the treatment of type-2-diabetes.

1.4 Therapeutic Obstacles and Opportunities

NRs control many aspects of biology including development, reproduction, and homeostasis through target gene activation. The ability to modulate their activity using fat-soluble molecules makes them extremely attractive drug targets. As our understanding of NR signaling increases, so does our appreciation of the complexity of their regulation. It is possible that the management of diseases in the future will include therapies that not only target NRs, but also co-regulator proteins and signaling pathways that are critical in the modulation of their function.

PPARs are the targets of some commonly used drugs in the treatment of hyperlipidemia and type-2-diabetes. Activation of PPARα by fibrates causes the up-regulation of genes involved in the β-oxidation of fatty acids. This results in the decreased synthesis of triglycerides and decreased LDL secretion by the liver [67]. Glitazones such as rosiglitazone and pioglitazone are PPARγ agonists. PPARγ is known to regulate glucose homeostasis and adipogenesis, making it an attractive target for the treatment of type-2-diabetes. However, recent evidence has indicated an increased risk of heart attacks with rosiglitazone (marketed as Avandia) and the FDA released a safety alert on the drug in May 2007 [209]. Further research surrounding the signaling events and co-regulator proteins that affect PPARγ activity

in multiple tissues may be useful in separating the therapeutic effects from the toxic effects of drugs like rosiglitazone.

One therapeutic challenge and opportunity in development of drugs that target NRs are selective therapeutic modulators (SRMs). SRMs are NR ligands that exhibit agonistic or antagonistic activity in a cell- or tissue-dependent manner. The classic SRM is tamoxifen, which can selectively activate or inhibit ERs and is commonly used in the treatment of breast cancer. Tamoxifen exhibits agonist (estrogen-like) activity in uterus and antagonist (anti-estrogen-like) activity in breast [210]. SRM-induced alterations in the conformation of NRs may affect the ability of the receptor to bind to co-regulators or to be phosphorylated. The expression profile of specific co-activator proteins and co-repressor proteins in a given cell type may affect the relative agonist -vs- antagonist activity of SRMs. However, as evidenced in this chapter, it is likely that cellular signaling events contribute to SRM activity due to altered activation, binding, and localization of co-regulator proteins, as well as NRs. Increased understanding of the effect of cellular signaling on NRs and their co-regulator proteins has the potential to aid in the process of discovery of novel SRMs and the development of new and more effective drug therapeutics.

Most NRs regulate a myriad of target genes that control multiple processes. One of the challenges in designing NR agonists is separating the desired therapeutic effects from the undesirable side effects. For example, the functional ability of LXRs to promote reverse cholesterol transport, improve glucose tolerance, and alleviate inflammation makes them attractive drug targets for the treatment of metabolic and

inflammatory diseases. However, the finding that first generation synthetic ligands of LXR increase hepatic lipogenesis and plasma triglyceride levels is a therapeutic obstacle that needs to be overcome [211]. The increase in hepatic lipogenesis has been attributed to the LXR mediated induction of SREBP-1C, therefore an agonist designed to increase reverse cholesterol transport but not to induce SREBP-1C may be a more effective therapy. Loss of LXR results in the increased expression of *ABCA1* and decreased expression of *SREBP-1C* suggesting that LXRs interact differentially with the transcriptional machinery on either promoter. A better understanding of the differential mechanisms and signaling pathways that interface with LXR during activation of specific target genes may provide insight into the design of a selective agonist or may present new drug targets.

Understanding of the signaling mechanisms that interface with NRs could also be useful in modulating the effect of a receptor without directly targeting it, or in the development of therapeutic molecules that only induce specific NR-target genes. There are multiple areas of potential therapeutic usefulness for FXR modulators such as cholestatic disorders, fatty liver disorder, or metabolic and inflammatory diseases. However, activation of FXR induces a complex physiological response that may lead to undesirable side effects in addition to the beneficial response. For example, the use of an FXR agonist in the regulation of glucose homeostasis may also result in the inhibition of bile acid synthesis and impact cholesterol excretion. Therefore, the identification of selective bile acid receptor modulators (SBARMs) may be necessary to target specific groups of genes modulated by FXR. Additionally, the identification

of how signaling pathways intersect with and modulate FXR may provide additional therapeutic opportunities that don't target FXR itself. For example, FGF19 or FGFR4 may prove to have interesting therapeutic potential in cholesterol and bile acid regulation.

The xenobiotic receptors PXR and CAR may have useful implications in the treatment of cholestatic liver disease. However, it has been hypothesized that unwanted activation of PXR is responsible for nearly 60% of all drug-drug interactions. Due to their promiscuous nature PXR and CAR are capable of modulating a number of genes in response to many different ligands. PXR and CAR activation by a specific drug results in the in the increased metabolism of not only that drug, but other drugs that may be in the system as well. In order for PXR and CAR to be effective therapeutic targets, the activation of a potential therapeutic-target gene must be separated from the activation of genes involved in drug metabolism. A better understanding of the co-regulator proteins and signaling pathways that interface with PXR and CAR may provide alternative drug therapies toward that end. In addition, pharmaceutical companies commonly screen for PXR activation by drug candidates in rodent and human species in order to avoid future drug-drug interactions. However, there is a significant species-specific response of PXR- and CAR-target genes with respect to activating ligands and signaling pathways. Understanding the signaling pathways that affect these two receptors may also be useful in the development of more accurate activation assays in order to predict and prevent unwanted and potentially lethal drug-drug interactions.

1.5 Conclusion

It is clear that multiple signaling pathways and phosphorylation events affect NR-mediated signaling. They modulate protein-protein interactions, sub-cellular localization, DNA-binding, protein stability, and transactivation capacity. The situation is further complicated by the fact that many NR cofactor proteins are themselves modulated by signaling pathways and phosphorylation events that affect their intrinsic and recruited enzymatic activities. Further investigation into the role of cell signaling pathways in NR-mediated transcription, and into signaling pathway crosstalk will be necessary to fully understand the functional implication of these signaling events. In addition, further characterization of these processes will likely lead to the development of novel and selective therapeutic molecules for a multitude of indications.

1.6 References

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Chapter 2: An Introduction to PXR Signaling

2.1 Historical Perspective

2.1.1 Regulation of Cytochrome-P450's by Diverse Compounds

All organisms are exposed continuously and unavoidably to foreign compounds, or xenobiotics, which are either ingested in the diet, inhaled, or otherwise absorbed. Normal homeostasis therefore requires the detoxification and elimination of these compounds from the body that are harmful when they accumulate to toxic levels. These compounds include both manufactured and natural compounds such as prescription and non-prescription drugs, pesticides, pollutants, industrial chemicals, alkaloids, pyrolysis products from cooked food, and other toxins that are generated from molds, plants and animals. The lipophilic property that enables many of these compounds to be absorbed is also an obstacle to their elimination. Consequently, their elimination is dependent upon their conversion to more polar derivatives that can be readily excreted. This process is known as biotransformation. Biotransformation is accomplished through the concerted action of the phase I oxidative cytochrome-P450 (CYP) enzymes, the phase II conjugating enzymes, and the membrane transporter proteins in the entero-hepatic system.

Members of the CYP family of heme-containing monooxygenases often catalyze the first enzymatic step in the clearance of lipophilic substrates. Members of the CYP1A, CYP2C, CYP2D, CYP2E1, and CYP3A subfamilies of drug metabolizing enzymes are of particular interest in this respect because of the structural diversity of their substrates and their relatively high abundance in liver and

intestine [1]. One long-standing observation is that the expression of the genes encoding many CYP isoforms, notably *CYP1A*, *CYP2B*, and *CYP3A*, can be dramatically induced by exposure to certain prescription drugs and other xenobiotic compounds. Frequently, compounds that induce the expression of a particular CYP enzyme are also a substrate for that particular CYP, thus providing a compensatory regulatory mechanism that amplifies the detoxification pathway during prolonged periods of xenobiotic challenge [2].

During the 1950's it was discovered that aromatic hydrocarbons (e.g., 3-methyl-cholanthrene) and barbiturates (e.g., phenobarbital (PB)) regulate the activity of different classes of CYP enzymes [3, 4]. Additionally, various types of xenobiotics including glucocorticoids (e.g. dexamethasone) and the perioxisome proliferators (e.g. clofibric acid) were shown to induce distinct classes of CYP enzymes. In fact, for many years CYP enzymes were biochemically classified based on the type of compound that induced their activity in liver. In the early 1970's, Hans Selye and colleagues introduced the concept of 'catatoxic steroids'. Catatoxic agents, such as the synthetic steroid pregnenolone 16α-carbonitrile (PCN), protect the liver of rodents against the effects of subsequently administered toxic substances by inducing CYP enzymes as well as other detoxifying enzymes [5, 6]. However, the mechanism for this type of induction and hepato-protection remained unknown for several decades.

In the early 1980's, the PCN-inducible CYP3A enzyme (known then as P-450PCN), was purified and shown to be distinct from previously characterized CYP

isoforms [7]. Further analysis of the gene encoding this CYP established it as a member of a distinct subfamily of P-450 genes [8, 9]. Biochemical studies in primary cultures of rat hepatocytes demonstrated that induction of this enzyme by PCN, originally thought to be a glucocorticoid receptor- (GR) mediated response, was in fact distinct from the classical GR signaling pathway. Guzelian and colleagues demonstrated that in addition to PCN, *CYP3A* gene expression was also inducible in rat hepatocytes by the glucocorticoid- dexamethasone [10, 11]. However, the kinetics of induction of the prototypical GR-target gene, tyrosine aminotransferase (TAT), did not coincide with the increases in *CYP3A* gene expression in that it took nanomolar dexamethasone concentrations to induce *TAT* expression but micromolar dexamethasone concentrations were required to induce *CYP3A* gene expression. A comparison of the effects of several steroid compounds supported this suggestion, because the concentration-response profiles of the compounds tested for *TAT* induction and *CYP3A* induction were completely different. Furthermore, three known GR antagonists (PCN, α-methyl-testosterone, and progesterone) repressed dexamethasone-mediated increases in *TAT* gene expression, but enhanced the induction of *CYP3A* gene expression [11-13]. These seminal studies convinced the research community that a novel receptor was implicated, but many years passed before the receptor involved in regulating drug-inducible *CYP3A* gene expression was identified.

In the intervening years it became increasingly clear that in rodents, the *CYP3A* subfamily of hepatic genes are inducible by a myriad of structurally diverse

compounds. For example, steroid compounds such as PCN, dexamethasone, betamethasone, hydrocortisone, mifepristone (RU486), α-methyl-prednisolone, dehydrorpiandrosterone, and spironolactone; the barbiturate PB; the anti-fungal drug clotrimazole; the antibiotic triacetyloleandomycin; the organochlorine-containing pesticides trans-nonachlor and γ-chlordane; the calcium channel antagonist nicardipine; the 11β-hydroxylase inhibitor metyrapone; and polychlorinated biphenyls were all found to be potent inducers of *CYP3A* gene expression in rodents [14-24]. It also became clear that there are distinct differences in the types of compounds that induce *CYP3A* gene expression in rodent hepatocytes when compared with human hepatocytes.

2.1.2 Species Differences in Response to Inducing Agents

It is a long-standing observation that the induction of *CYP3A* gene expression exhibits a species-specific pharmacology. For example, classical biochemical studies demonstrated that rifampicin was an efficacious *CYP3A* inducer in rabbit, but had little effect in rat, whereas PCN, an inducer in rat, failed to induce in rabbit. PB had a moderate effect in all species tested, suggesting that it may be acting by an alternate mechanism [23]. Species differences were also well documented in the response of human hepatocytes to *CYP3A* inducers. The expression of *CYP3A* in humans and rabbits is strongly induced by rifampicin, whereas rodent *CYP3A* genes are poorly induced by this drug. On the other hand, PCN is a potent inducer of rodent *CYP3A* genes, but a weak inducer in human and rabbit [16, 25-28]. These data indicated that

there were likely important species-specific differences in the receptor that regulated *CYP3A* gene expression.

In humans, CYP3A4 is the predominant CYP isoform expressed in liver and it is involved in the metabolism of more than 50% of all prescription drugs. The induction of *CYP3A4* gene expression by xenobiotic compounds is well documented and represents the basis for an important class of drug-drug interactions in which administration of one drug accelerates the metabolism of a second clinically prescribed drug [29]. Therefore, since rodents are commonly used in pre-clinical drug development studies in the pharmaceutical industry, the elucidation of the mechanisms underlying species-specific *CYP3A* expression was deemed to be extremely important for the development of safer therapeutic treatments for humans.

2.1.3 Xenobiotic Response Elements (XREs) in the Rat Cyp3A1 Promoter

To investigate the mechanism of non-classical glucocorticoid induction of the rat *Cyp3A1* gene, Burger *et al.* fused a 1.5 kb fragment of the 5'-flanking region to a reporter gene. Deletion analysis identified a specific 164 base pair region of the promoter (bases -220 to -56) that mediated both dexamethasone- and PCN-responsiveness and that does not contain an identifiable glucocorticoid response element [12]. Further analysis of this region led to the identification of three sites that contained binding motifs for members of the nuclear receptor (NR) superfamily; sites A, B and C (Figure 2-1) [30, 31].

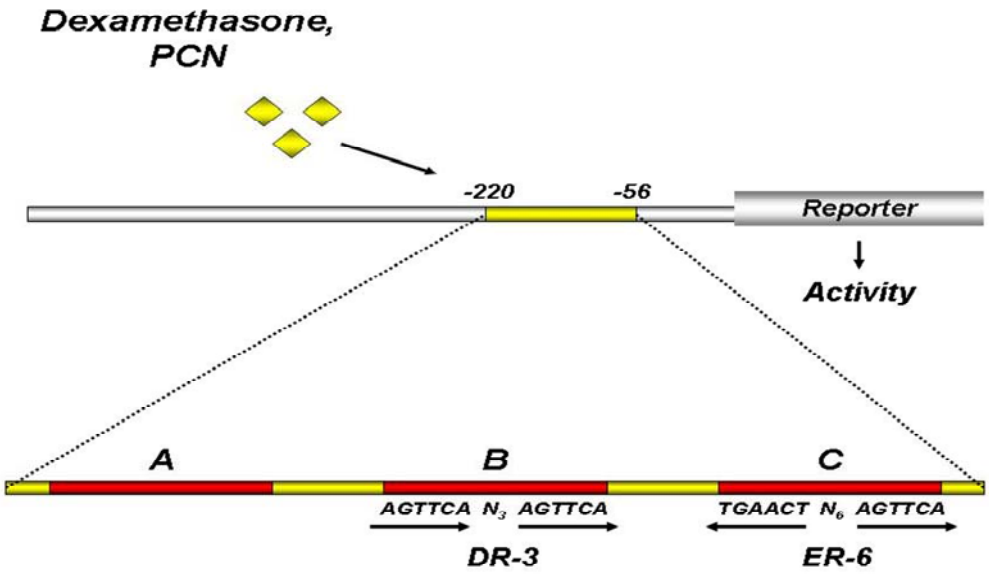

Figure 2-1. Identification of xenobiotic response elements in the *CYP3A* promoter. A 1.5 kb fragment of the 5' flanking region of the *Cyp3a* gene was fused to a reporter gene. Deletion analysis identified a fragment (-220 to -56) that mediates dexamethasone and PCN responsiveness. This region does not contain a glucocorticoid response element, but does contain three NR binding sites (A, B, and C). Mutation analysis demonstrated that sites B and C are necessary for micromolar glucocorticoid responsiveness. These sites contain a DR-3 and ER-6 of the consensus AGTTCA PXR-response element.

Site-directed mutation analysis demonstrated that sites B and C were necessary for maximum glucocorticoid responsiveness. Site B contained a direct repeat of the NR consensus binding site AGTTCA separated by three nucleotides (DR-3) and site C contained an everted repeat separated by 6 nucleotides (ER-6). Neither site was capable of interacting with GR, but formed a common protein complex with nuclear extracts prepared from a rat hepatoma cell line [30-32]. Disruption of site B resulted in a complete loss of dexamethasone- and PCN-responsiveness. In addition, although sites A and C appeared to have little ability to modulate the activity of reporter genes by dexamethasone and PCN, they were required for maximal induction of *Cyp3A1* reporter gene expression [31-33].

In an experiment using the 5'-flanking sequences of rabbit, rat and human *CYP3A* genes and hepatocytes from rats and rabbits, Barwick *et al.* demonstrated that the inducibility of *CYP3A* genes was determined by the cellular environment rather than by the structure of the gene [34]. When reporter constructs containing the 5'-flanking sequences of human, rat, or rabbit *CYP3A* were transfected into rat hepatocytes, all three were induced by dexamethasone and PCN, but not by rifampicin. However, in rabbit hepatocytes, rifampicin and dexamethasone, but not PCN, transactivated the *CYP3A* reporter genes [34]. This study further supported the idea that there are likely species-specific differences in the receptor that regulates *CYP3A* expression.

2.2 Cloning and Characterization of PXR

2.2.1 Discovery and Cloning of PXRs

NRs are one of the largest groups of transcription factors with 48 members in the human genome that regulate diverse biological processes including metabolism, homeostasis, development, and reproduction [35]. NRs can be loosely divided into two groups based on their ligand status. The first group is composed of receptors for classical endocrine hormones. NRs in the second group are referred to as orphans. Orphan receptors are cloned based on DNA sequence homology to classical endocrine receptors. However, at the time of their cloning, nothing was known about their physiological ligands. Pregnane x receptor (PXR, NR1I2) is a member of the NR1I subfamily which includes the vitamin D_3 receptor (VDR, NR1I1) and constitutive androstane receptor (CAR, NR1I3).

All members of the NR superfamily share several conserved structural domains that are essential for receptor function (Figure 2-2) [36]. These domains include a highly variable N-terminal domain, a central DNA-binding domain (DBD), and a C-terminal ligand-binding domain (LBD). The highly conserved DBD is approximately 70 amino acids in length. The DBDs of NRs each contain two α-helices and two zinc fingers. Each zinc finger contains four highly conserved cysteine residues that chelate a zinc atom. The DBD is critical for the specificity of response element recognition and imparts critical receptor dimerization properties. While some NRs function as monomers, most NRs are active as dimers; either as homodimers, or as heterodimers with retinoid x receptor (RXR) [37]. The DBD is

connected to the LBD by a hinge region (H) that contains a nuclear localization signal (NLS). The LBD is approximately 250 amino acids in length and folds to form a hydrophobic pocket suitable for ligand binding. The LBD also contains transcriptional activation motifs including the well-characterized activation fuction-2 (AF-2) helix in the extreme C-terminal portion. Upon ligand binding, the AF-2 domain undergoes a conformational change and creates a new surface to interact with co-activator proteins and activate transcription [38]. The N-terminal region of NRs is highly variable in sequence and length, but all contain an activation function-1 (AF-1) domain that acts independently of ligand and is responsive to cell signaling pathways [39, 40].

Figure 2-2. Nuclear receptors share a high degree of structural homology. Members of the NR superfamily share four modular domains: a highly variable N-terminal region that in some NRs harbors a ligand-independent activation function (AF-1), a DNA-binding domain (DBD) that contains two zinc finger motifs, a flexible hinge domain (H), and the ligand-binding domain (LBD) that contains a ligand-dependent activation function (AF-2).

Pregnane x receptor (PXR, NR1I2) was originally identified on the basis of its DNA sequence homology with other NRs in the expressed sequence tag database. The full-length mouse PXR (mPXR) cDNA was characterized in 1998 using the expressed sequence tag to screen a mouse liver cDNA library, and the receptor was named PXR based on its activation by pregnane (21-carbon) steroids [41]. Shortly after its discovery, PXR was classified as a broad specificity receptor that is activated by a wide variety of drugs and xenobiotic compounds as a heterodimer with RXRα. Upon ligand binding the PXR-RXR heterodimer binds to multiple sites on the *CYP3A* promoter and activates gene expression, at long last providing the molecular basis for the induction of *CYP3A* gene expression by xenobiotics [41, 42]. At approximately the same time the human PXR (hPXR) was cloned by three separate groups. One group identified hPXR in studies aiming to identify homologues of the *Xenopus laevis* benzoate x receptor (BXR) [43]. Another group established hPXR as a homologue of mPXR [42]. In addition, a parallel computational search approach identified hPXR [44]. Since then, PXR has been cloned and functionally expressed from many other species including monkey, dog, rabbit, and rat as well as closely related receptors in chicken, frog and zebrafish [45-49].

2.2.2 PXR Expression Patterns

The mRNA encoding the PXR protein is highly expressed in the liver, small intestine and colon in human, rabbit, rat and mouse [41-44, 46, 48, 49]. Markedly, these are the same tissues where the *CYP3A* gene is highly expressed, and *CYP3A* expression is induced in these tissues following xenobiotic exposure. Lower levels of

Pxr gene expression have been detected in stomach, kidney and lung of rodents [24, 41, 50]. Studies aimed at characterizing the expression of the *Pxr* gene during the perinatal period in mice detected expression in the ovary and uterus of female mice, as well as in the placenta throughout gestation [51]. In rats, the *Pxr* mRNA is expressed in the capillaries and endothelial cells that comprise the blood-brain barrier, and *Pxr* expression is increased by dexamethasone in a dose-dependent and reversible manner [52, 53]. Finally, in humans, the *PXR* mRNA has been detected in both normal and neoplastic breast tissue [54], as well as in peripheral blood mononuclear cells (PBMCs) [55]. In addition, a study of Japanese subjects has shown that the amounts of PXR protein in the liver and intestine reach maximal levels in young adults (15-38 years of age); subsequent decreased expression levels are detected with aging [56].

Relatively little is known about the mechanism by which the expression of the *PXR* gene is regulated. The promoter that drives expression of the *PXR* gene has not been well characterized. However, two factors have been identified in the endogenous regulation of *PXR* gene expression: the GR and the liver-specific transcription factor hepatic nuclear factor 4α (HNF-4α). Notably, dexamethasone increased *Pxr* mRNA expression in the rat hepatoma-derived cell line H4IIE. This effect appears to be mediated through GR because it required nanomolar concentrations of dexamethasone, and the effect was opposed by the GR antagonist RU486 [57]. Similar results were obtained in another study utilizing human hepatocytes in which treatment with dexamethasone, hydrocortisone, or prednisalone

stimulated the expression of *PXR* mRNA [58]. This increase in *PXR* gene expression may contribute, in part, to the stimulation of *CYP3A* gene expression by dexamethasone. Interestingly, expression of *Pxr* mRNA is increased nearly 50-fold in mouse liver and ovary during pregnancy, suggesting that expression of the *Pxr* gene may be stimulated by other hormones as well [51]. The extent to which these phenomena are conserved across species is not currently known.

The *Hnf-4α* gene is essential for the normal development of murine liver. In fact, it is not possible to generate non-conditional HNF-4α-null mice, because deletion of the *Hnf-4α* gene in mice produces a non-viable embryo. However, mice with HNF-4α-null livers were generated by aggregating tetraploid embryos with HNF-4α -/- ES cells. When gene expression was analyzed in the livers of mice generated using this method, *Pxr* mRNA was markedly absent [59]. In another study, HNF-4α-null fetal hepatocytes were generated by Cre-mediated inactivation of the *Hnf-4α* gene. Expression of *Cyp3a11* and *Pxr* were suppressed by the inactivation of *Hnf-4α*. In addition, a functional HNF-4α-response element was characterized in the *Pxr* promoter, and it is required for *Pxr* gene activation in fetal hepatocytes [60]. In adult HNF-4α-null mice generated using a floxed *Hnf-4α* allele, basal expression of an *in vivo* transfected *CYP3A* reporter gene construct was 50% of that observed in wild-type mice, however the HNF-4α-null mice were still able to respond to treatment with PCN and induce PXR-target gene expression. Moreover, in adult HNF-4α-null mice it was *Car* expression that was suppressed, not *Pxr* expression

[61]. Without further data, it is difficult to determine the extent to which the effect HNF-4α has upon *Pxr* gene expression is either direct or indirect in embryonic or adult tissues.

More recently, Gibson *et al.* performed an *in silico* analysis of the human *PXR* promoter in an attempt to identify positive and negative regulatory elements [62]. The analysis identified a peroxisome proliferator activated receptor α (PPARα) response element located at -1318 to -1338 base pairs upstream of the transcription start site. Furthermore, expression of the *PXR* mRNA was increased by PPARα in reporter gene studies and mutation of the PPARα response element ablated that expression [62]. These results are consistent with a previous report that PPARα agonists' clofibrate and perfluorodecanoate (PFDA) induce *PXR* expression in rat hepatocytes [63].

Gibson *et al.* also demonstrated that the human *PXR* proximal promoter is activated by forkhead transcription factor A2 (FOXA2, also known at HNF-3β) [62]. Additional studies have shown that FOXA2 is recruited to a region of the mouse *Pxr* promoter between -167 and -193 during fetal liver development, and that novel single nucleotide polymorphisms (SNPs) in the human *PXR* promoter as well as in the first intron affect putative binding sites for FOXA2 and affect subsequent PXR-target gene expression [64, 65].

2.2.3 Cross-species Variation of PXR

NR genes typically show a high degree of sequence homology across species. For example, amino acid sequence identities between human and mouse NR

orthologues are typically greater than 95% in the DBD and greater than 85% in the LBD [66]. A study comparing human, mouse and rat genomes and another study comparing human, mouse and chimpanzee genomes revealed that NRs have been subjected to negative evolutionary selection [66, 67]. However, the two notable exceptions in the NR superfamily are the LBDs of the 'xenobiotic sensor' receptors PXR and CAR [68].

The PXR-LBDs are much more divergent across species when compared with other NRs (Figure 2-3). The LBD of human PXR shares an amino acid identity of approximately 75% when compared with rodent sequences. Furthermore, the human PXR-LBD shares 50% identity with the zebrafish and chicken PXR-LBD sequences. These LBD comparisons represent the lowest percent identity shared across species in the NR superfamily, whose members typically have comparable identities between species that are approximately 10-15% higher [47, 66]. The relatively large degree of divergence that occurs in the PXR-LBD amino acid sequence across species indicates that *PXR* genes have evolved to exhibit functionally and significantly altered ligand-activation profiles, likely as a protective adaptation to differences in exposure to various toxic compounds in their respective environments.

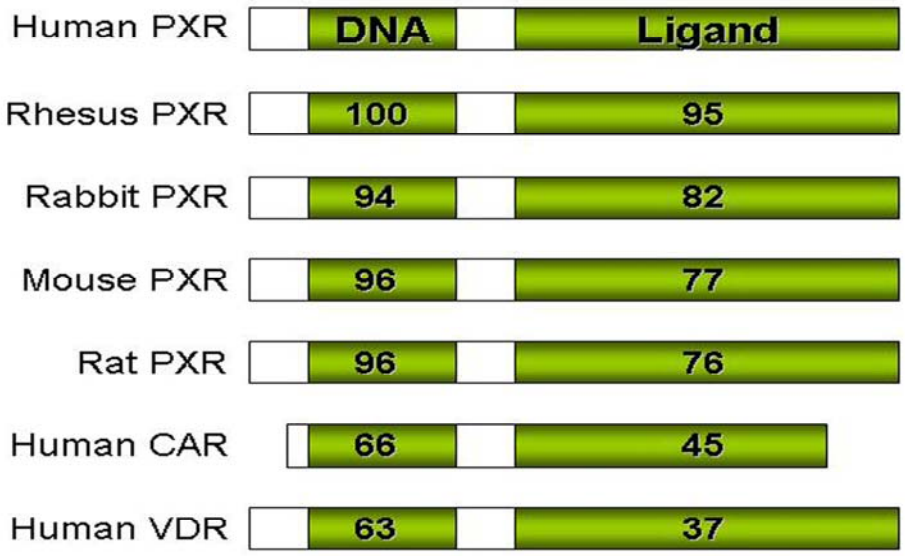

Figure 2-3. Sequence comparison of PXR across species. Sequences of PXR from various species and other NR1I family members are aligned. The similarity is expressed as percentage amino acid identity in the DBD and LBD.

Traditionally, compounds that activate NRs have been identified using cell based reporter gene assays. The receptor expression plasmid typically encodes the full length receptor or a chimera of the NR-LBD fused to the DBD of the yeast transcription factor- GAL4. The reporter gene contains binding sites for the full length or chimeric receptor upstream of a gene that encodes an easily quantifiable protein. Using this strategy, numerous PXR-activating compounds have been identified across various species. For example, mouse and rat PXR were activated by the *CYP3A* inducer PCN, whereas PCN had little effect on human and rabbit PXR. On the other hand, rifampicin activated human and rabbit PXR but had virtually no effect on the mouse and rat receptors (Figure 2-4) [42, 43, 46, 48].

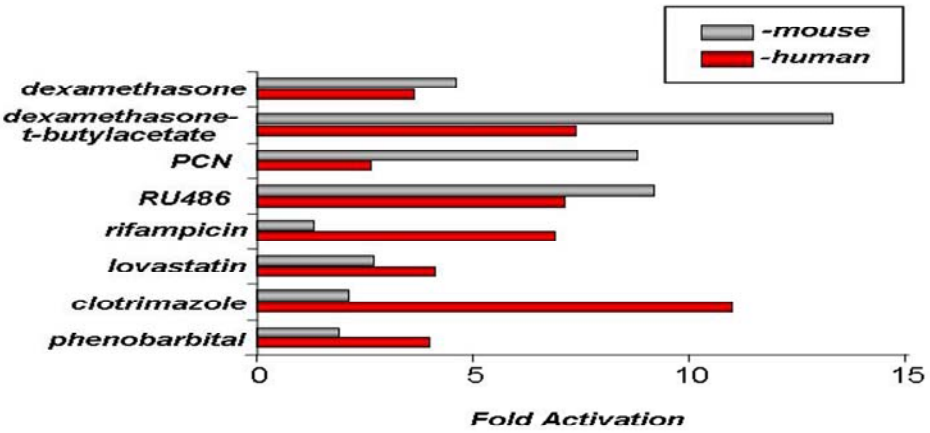

Figure 2-4. Differential activation of mouse and human PXR by ligands. Human and mouse PXR are activated by ligands in a species-specific manner. PCN is a strong activator of mouse PXR but has little effect on human PXR, whereas rifampicin is a strong activator of human PXR but has little effect on mouse PXR.

2.2.4 Inter-individual Variability of PXR Activity in Humans

The expression level of *CYP3A4* mRNA in the liver can vary by more than 50-fold between individual humans, and may account for much of the observed inter-individual variability in the metabolism of drugs. However, it was recently discovered that very few polymorphisms exist in the *CYP3A4* gene and promoter [69]. More recent studies have shown that *PXR* expression in human liver is subject in inter-individual variability [70]. In addition, several allelic variations of the human *PXR* gene are found that result in single amino acid substitutions, and the mRNA encoding the PXR protein is subject to a number of alternative splice variants.

In comparison with other human genes, relatively few SNPs are observed in the *PXR* gene. However, a study in subjects of Caucasian and African origin identified 40 SNPs in the *PXR* gene, including seven that coded for missense mutations. Four of the missense mutations (R122Q, V140M, D163G, and A370T) displayed altered basal and altered rifampicin-inducible gene activation profiles in cell-based reporter gene assays. In particular, R122Q residing in the DBD of PXR markedly decreased its DNA binding activity *in vitro*. However, this alteration has only been found as a heterozygous allele with the other normal *PXR* allele *in vivo*, resulting in no detectable change in activity. V140M, D163G, and A370T all change residues in the PXR-LBD. In reporter gene studies, D163G had reduced basal and ligand-induced activity, whereas V140M and A370T exhibited slight increases in basal activity [71-73]. Additional amino acid changes have been identified in Japanese subjects (R98C, R381W, and I403V) and Chinese subjects (Q158K) that

result in a decrease in PXR activity [74, 75]. The allelic frequency of each of these polymorphisms is less than 3%, which indicates that they likely do not account for much inter-individual variability.

In addition to single amino acid changes, the *PXR* mRNA is subject to alternative splicing that may be a significant source of inter-individual variability. Human PXR exhibits alternative splicing in exon 5; yielding slightly different LBDs designated hPXR.1 and hPXR.2 (with hPXR.2 lacking 37 amino acids in exon 5) [73]. In a study of tissue-specific mRNA expression, two more splice variants were identified; hPXR.3 (with a 41 amino acid deletion) and hPXR.4 (with a 71 base pair deletion leading to a frameshift and premature termination) were detected [76]. An additional form of PXR incorporates an alternative exon 1 (encoding 39 additional amino acids at the N-terminus) and appears to be as effective as hPXR.1 at inducing *CYP3A* gene expression [77, 78]. The expression of hPXR.1 is variable between individuals, but is consistently higher in females than males, correlating with the increased expression of *CYP3A* mRNA in females. The hPXR.2 variant is accounts for 6-7% of hPXR transcripts, and the other isoforms are expressed at negligible levels [76].

2.2.5 Structure of PXR

PXR contains all the conserved domain structures of classical NRs. Most NRs interact with their ligands in a highly selective manner. In contrast, PXR serves as a 'xeno-sensing' receptor and is activated by diverse array of lipophilic compounds. Despite this relatively promiscuous ligand-binding profile, species-

specific differences are observed in the activation profiles between PXR orthologs. Determination of the three-dimensional crystal structure of the human PXR-LBD has shed some light on the molecular basis of these properties.

Crystal structures of the hPXR-LBD have been determined alone, in complexes with several xenobiotics, and with a peptide fragment of the co-activator steroid receptor co-activator-1 (SRC-1) [79-83]. Similar to most NR-LBDs, the PXR-LBD contains a three-layer sandwich of α-helices. However, while most NRs contain a short two- or three-stranded β-sheet, the PXR β-structure is extended to a five-stranded anti-parallel structure [82]. The PXR ligand-binding pocket is large, flexible and capable of varying in volume between 1,150 and >1,600 Å. Thus, PXR is comparable with the largest known NR ligand-binding cavity, which is 1,619 Å for the fatty acid binding receptor peroxisome proliferator activated receptor γ (PPARγ) [84]. The PXR ligand-binding cavity is predominately hydrophobic, but contains eight polar residues that commonly interact with ligands and are distributed evenly throughout the surface of the pocket [79, 82]. Another interesting feature of the PXR ligand-binding pocket is an expandable pore that is created by a flexible loop (amino acids 309-321), which opens to expose more hydrophobic residues and to accommodate larger ligands [82]. The smooth, elliptical shape and the flexible nature of the PXR ligand-binding pocket undoubtedly contribute to its unusual broad-specificity of ligand binding.

An additional structural feature of PXR is that during ligand binding, AF-2 moves to a conformation that favors interactions with co-activator proteins. For

example, in the absence of SRC-1 peptide, the small ligand SR12813 was shown to bind to the PXR-LBD in numerous distinct orientations at once. However, when the –LXXLL- motif of the co-activator SRC-1 peptide is bound to the α2 helix of AF-2 the agonist shifts to a single binding orientation [80]. Therefore, since co-activator proteins work in concert with ligand-binding in this manner, the ligand-induced response and specific repertoire of PXR-target genes that are activated is also likely dependent upon which co-activator protein(s) is present.

Finally, there are also structural clues in the PXR protein that elucidate the molecular basis of species-specificity in the ligand response. Two key residues in the ligand-binding pocket of hPXR, G285 and H407, are not conserved across species. Site-directed mutagensis studies were performed in which G285 was mutated to I and H407 was mutated to G, the equivalent residues in mouse. G285I indeed did show increased responsiveness to PCN and decreased responsiveness to rifampicin, while the H407G mutant had little effect [85]. Site-directed mutagenesis has also been used to examine the differences in ligand-binding between human and rat. Mutation of F305 to L conferred rifampicin sensitivity and abolished PCN responsiveness on the rat receptor. The reverse mutation of the human residue L308 to F reduced the receptors affinity for rifampicin but not its ability to undergo rifampicin-mediated activation [86]. In a study using rat-human PXR chimeras, replacement of a region (amino acids 306-326) of the human gene with the rat gene converted the reporter into a PCN-responsive state [87]. Similarly, when an equivalent residue in mouse PXR (F305) was mutated to the residue found in rabbit (V305), the PXR response to

PCN was decreased by 100-fold and the response to rifampicin was increased 3-fold [79]. The mutated residues are located in a flexible loop that forms the entrance to the ligand-binding pocket; therefore the mutation may affect the ability of this loop to rearrange in response to ligand binding.

2.2.6 PXR is Activated by a Diverse Set of Ligands

PXR is activated by a broad range of lipophilic compounds including a myriad of synthetic and endogenous steroids, certain bile acids, and a variety of drugs and plant products (Figure 2-5). In contrast to the classic steroid hormone receptors, high-affinity (sub-nanomolar) ligands for PXR have not been discovered. For example, the lowest EC_{50} values of steroids that activate PXR are low-micromolar, generally two to three orders of magnitude higher than concentrations found in circulating plasma [46, 47]. Even the highest affinity PXR ligands such as hyperforin, the active component of St. John's wort, only have binding affinities in the mid-nanomolar range [88, 89].

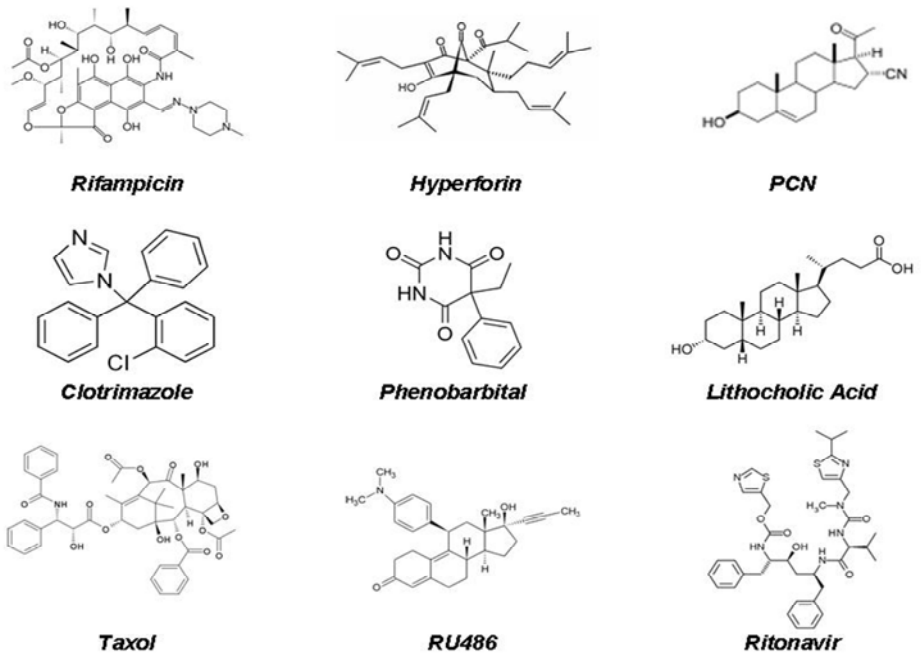

Figure 2-5. PXR activity is regulated by structurally diverse ligands.
Represented here are xenobiotic compounds of various shapes and sizes that activate either human or mouse PXR.

PXR is activated by a variety of natural and synthetic steroids such as pregnelenone, progesterone, several phyto-estrogens, dexamethasone and antiglucocorticoids [41-44, 46, 90]. Some bile acids including lithocholic acid (LCA) and ursodeoxycholic acid have also been shown to activate PXR [91-93]. The list of drugs and natural compounds that activate PXR is rapidly expanding. Xenobiotics that activate PXR include the previously established *CYP3A* inducers PB, metyrapone, clotrimazole, spironolactone and tans-nanochlor [42, 90, 94, 95]. Other PXR activators include the HIV protease inhibitor ritonavir [96]; the calcium channel blocker nifedipine [97]; the anticancer drugs tamoxifen and paclitaxel [98]; the anti-diabetic drug troglitazone [46]; the cholesterol lowering statins and SR12813 [46]; and endocrine disruptors bisphenol A and nonylphenol [99, 100]. In addition, PXR has also been shown to be activated by a variety of compounds found in natural products including St. John's wort, gugulipid, kava kava, Coleus forskolii, tian xian and ginko biloba [101-103]. While the majority of publications characterize novel PXR activators, there is also a growing list of PXR antagonists that includes ET-743 [104], polychlorinated biphenyls [105], ketoconazole [106], sulforaphane [107], coumestrol [108] and the HIV protease inhibitor A-792611 [109].

2.2.7 PXR-target Genes

PXR-selective ligands have been shown to stimulate genes involved in the oxidation (phase I), conjugation (phase II) and transport (phase III) of xenobiotics. The first genes shown to be regulated by PXR were *CYP3A* family members in mouse and human [41, 42]. In fact, over time, PXR has been termed the master regulator of

drug-inducible *CYP3A* gene expression. Additional phase I drug metabolism gene products regulated by PXR include numerous *CYP*s, aldehyde and alcohol dehydrogenases, carboxylesterases, and enzymes related to heme production and support of the CYP cycle such as aminolevulonic acid synthase and P450 oxidoreductase [80, 110, 111]. Phase II drug metabolism gene products regulated by PXR include UDP-glucuronosyl-transferases (*UGT*s), sulfotransferases (*SULT*s) and glutathione S-transferases (*GST*s) [80, 111-115]. Finally, phase III drug metabolism gene products regulated by PXR include numerous ATP-binding cassette membrane pumps of the multidrug resistant family (*Mdr*s and *Mrp*s) and organic anion transporting protein 2 (*OatP2*) [80, 93, 96, 104, 111, 116-118].

The list of genes that are regulated by PXR continues to grow, and now includes genes that are not only involved in xenobiotic metabolism, but also those involved in cholesterol and bile acid homeostasis and other cellular processes. For example, gene products known to be regulated by PXR include fatty acid and HMG coA synthases, *OATP*s and *CYP*s involved in cholesterol and bile acid metabolism [92, 119-121]. Additional PXR-target genes include *insig-1* (encoding a protein with antilipogenic properties) and *CD36* (encoding a free fatty acid transporter) [122, 123]. PXR also upregulates the target gene inducible nitric oxide synthase (*iNOS*), that is known to influence inflammation and apoptosis [124]. Finally vitamin K activates PXR and transcription of *Msx2*, an osteoblatogenic transcription factor [125]. A list of PXR-target genes is summarized in Table 1-1.

Table 2-1. Target genes of PXR. Listed here are target-genes that are regulated by the activation of either human or rodent PXRs.

Class	*Gene*	*Organism*
Phase I Enzymes	***CYP1A1, CYP1A2***	***Human***
	CYP2A6	***Human***
	Cyp2B1/2	***Rat***
	CYP2B6	***Human***
	Cyp2b10	***Mouse***
	CYP2C8, CYP2C9, CYP2C19	***Human***
	Cyp3A1/2	***Rat***
	CYP3A4, CYP3A7	***Human***
	Cyp3a11, Cyp3a13,Cyp3a44	***Mouse***
	Cyp3A23	***Rat***
	Cyp7a1	***Mouse***
	CYP24A1	***Human***
	Aldh1a1, Aldh1a7	***Mouse***
Phase II Enzymes	***Sult2a1***	***Mouse***
	SULT2A1	***Human***
	Ugt1a1	***Mouse***
	UGT1A1, UGT1A3, UGT1A4	***Human***
	Gsta1, Gsta4	***Mouse***
	GstA2	***Rat***
	Gstm1, Gstm2	***Mouse***
Transporters	***MDR1***	***Human***
	Mdr1a, Mdr1b	***Mouse***
	Mrp2, Mrp3	***Mouse***
	Oatp2	***Mouse***
	Oatp2	***Rat***
Accessory Proteins	***Alas1***	***Mouse***
	ALAS1	***Human***
	PAPSS2	***Human***
	Por	***Mouse***
Receptors	***AHR***	***Human***
	Car	***Mouse***
	Pxr	***Mouse***
Other	***iNOS***	***Human***
	MSX2	***Human***
	Insig-1	***Mouse***
	CD36	***Mouse***

2.3 Physiological Functions of PXR

2.3.1 Xenobiotic Metabolism and Liver Toxicity

As described above, PXR is activated by a myriad of xenobiotic compounds and regulates numerous genes involved in drug and xenobiotic metabolism. Induction of CYPs (phase I drug-metabolizing enzymes) is the most characterized system of PXR gene regulation. However, PXR is also involved in the regulation of other aspects of drug metabolism and excretion. PXR regulates the expression of genes encoding phase II drug metabolizing proteins including UGT, SULT, and GST enzymes that function to conjugate hydrophilic groups thereby increasing the water solubility of compounds [80, 111-115]. The PXR protein can also up-regulate the expression of transporters such as Bsep, Ntcp, OatP2, Mrp3, and Mdr2 [92, 126]. These drug transporter proteins promote the uptake of xenobiotics into the liver for subsequent metabolism by phase I and phase II enzymes, and then move the conjugated metabolites into an excretory pathway either into bile or back into blood for excretion via the kidney. In this manner, PXR activation increases clearance of xenobiotics by simultaneously increasing hepatic uptake, metabolism and excretion of numerous potentially toxic compounds (Figure 2-6). Therefore, PXR activation is a principle defense mechanism defending the body from toxic assault.

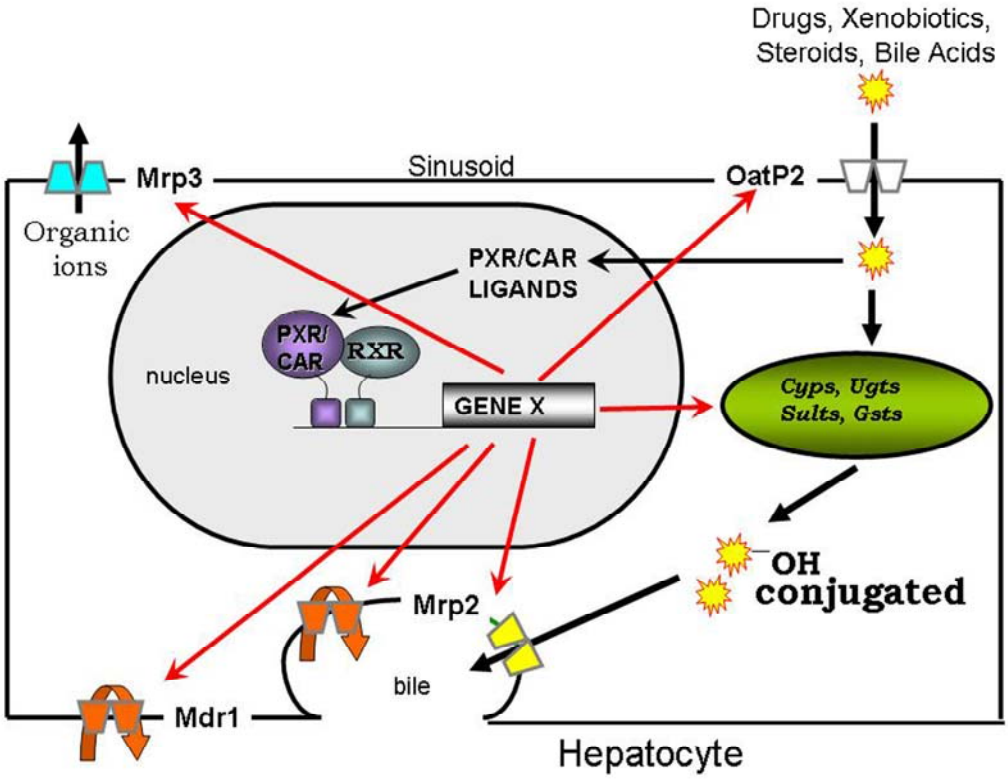

Figure 2-6. Mechanism of hepatoprotection by PXR. Xenobiotic and endogenous compounds enter hepatocytes where they are metabolized into more polar subtrates and excreted. Many of these compounds are PXR ligands. These ligands activate PXR and result in the upregulation of genes involved in xenobiotic biotransformation. In this manner PXR activation protects hepatocytes from toxic assault. However, PXR activation and subsequent upregulation of PXR target-genes also represents the basis for an important class of drug-drug interactions.

Occasionally, drug metabolizing enzymes produce chemically reactive metabolites that covalently bind to hepatic macromolecules resulting in liver toxicity [127]. Acetaminophen (APAP) toxicity represents one example of this type of toxicity. At high doses, CYP enzymes including CYP1A2, CYP2E1, and CYP3A4 convert APAP to a reactive quinine called N-acetyl-p-benzoquinone imine (NAPQI) that binds to cellular macromolecules and causes the production of reactive oxygen species [128, 129]. In fact, APAP toxicity is increased in humans and rodents by pretreatment with CYP inducers such as PCN [130, 131]. Since PXR is a master regulator of CYP3A in liver, its role has been investigated in APAP-induced hepatotoxicity. Pretreatment with the PXR activator, PCN, enhanced APAP-induced hepatic toxicity, as evidenced by increased serum alanine aminotransferase (ALT) levels and hepatic centrilobular necrosis, in wild-type but not in PXR-null mice. This was due to the fact that PXR-null mice had lower *Cyp3a11* expression levels and decreased NAPQI formation compared to wild-type mice treated with PCN [132]. Thus, the possibility of treating APAP toxicity by targeting PXR is an intriguing one. However, since PXR and CAR engage in significant crosstalk and regulate overlapping sets of target genes a suitable clinical inhibitor may be difficult to design.

2.3.2 Drug-drug, Herb-drug, and Food-drug Interactions

Although PXR plays a critical role in the protective response against xenobiotics, its activation also represents the basis for an important class of drug-drug interactions. It is clear that most drugs that induce expression of the *CYP3A* gene do so through the activation of PXR. In fact, many of the xenobiotics that activate PXR

are prescription drugs, including the antibiotic rifampicin, the HIV protease inhibitor ritonavir and the chemotherapeutic drugs paclitaxel and cisplatin, to name a few [93, 96, 98, 133-135]. Activation of PXR and the subsequent induction of *CYP3A* and other genes can result in the accelerated metabolism of other medications. The CYP3A4 enzyme alone is involved in the metabolism of >50% of all prescribed drugs. Therefore, drugs that activate PXR have the potential to reduce the therapeutic effectiveness of more than one-half of co-administered drugs. This phenomenon is a serious concern for patients taking multiple medications with small therapeutic indices as alterations in drug metabolic rates in patients can often have life-threatening consequences.

In addition to prescribed drugs, many natural products activate PXR and represent the basis for potential adverse drug reactions. Natural products are not regulated in this country, unlike prescription drugs. However, products such as St. John's wort, gugulipid, kava kava, Coleus forskolii, tian xian and ginko biloba have all been shown to activate PXR [88, 101-103]. Activation of PXR by St. John's wort is a classic example of this type of herb-drug interaction. St. John's wort is widely used as an over-the-counter treatment for a variety of indications including inflammation and mild depression. In patients, St. Johns wort enhances the metabolism of prescription drugs including oral contraceptives, the immunosuppressant cyclosporin, the HIV protease inhibitor ritonavir, the anticoagulant warfarin, and the cardiac glycoside digoxin [136-140]. Indeed, commercial preparations of St. John's wort activate PXR in cell-based reporter gene

assays and induce the expression of *CYP3A4* in primary human hepatocytes [88, 89]. Further analysis revealed that hyperforin is the chemical component of St. John's wort that binds to and activates PXR with high affinity [88, 89]. This provides a molecular explanation for the clinical reports that describe an interaction between prescription drugs and St. John's wort.

The knowledge that PXR activation is the basis for drug-drug and herb-drug interactions, as illustrated above, should aid in the development of safer medications. *In vitro* assays that detect PXR activity are readily available. Ideally, drug candidates would not activate PXR, and those that do can be replaced with compounds that have similar therapeutic efficacy, but lack the ability to activate PXR. For example, the cancer drug paclitaxel and its analog docetaxel have similar anti-neoplastic activity, but paclitaxel is a strong PXR activator, whereas docetaxel is not [104]. Another example is the anti-diabetic drugs troglitazone, pioglitazone and rosiglitazone. All three drugs are potent PPARγ agonists, but only troglitazone is also a PXR agonist [46]. In fact, troglitazone was later withdrawn from the market due to hepatotoxicity [141].

Dietary chemicals also have the capacity to activate PXR and represent the basis for a food-drug interaction. Dietary consumption of phytochemicals occurs as a part of vegetables, fruits, spices, flavoring agents, and beverages. Some phytochemicals including flavonoids, carotenoids, and terpenoids markedly activate PXR and induce the expression of *CYP3A* [142, 143]. Thyme, curcumin, resveratrol, and quercetin, are all common phytochemicals that activate PXR on the *CYP3A*

promoter [144-146]. Another group of compounds that activate PXR are plant estrogens. Phytoestrogens and mycoestrogens are plant-derived natural compounds that are ingested through diet. Some of the plant estrogens that have been shown to activate PXR include ferutinine, zearalenone, and the hops constituent colupulone [147-149]. On the other hand, the phytoestrogen coumestrol has been identified as a naturally occurring PXR antagonist [108]. In addition to naturally occurring dietary compounds, the food preservative tertiary butylated hydroquinone (tBHQ) also activates PXR [145]. The scope has also widened to include vitamins with the demonstration that vitamin E and vitamin K_2 are activators of PXR-mediated expression of target genes [150, 151]. Taken together, these data signal that patients should be aware of potentially dangerous drug-drug and supplement-drug interactions involving the activation of PXR.

2.3.3 Steroid Hormone Homeostasis

Recent studies have shown that PXR plays an important role in adrenal steroid hormone homeostasis. The concept of PXR as a potential endocrine disrupting factor may have implications in drug-hormone interactions. Activation of PXR markedly increases plasma concentrations of corticosterone and aldosterone, the primary glucocorticoid and mineralocorticoid in rodents. The increased levels of corticosterone and aldosterone were associated with activation of adrenal steroidogenic enzymes, including CYP11A1, CYP11B1, CYP11B2, and 3β-hydroxysteroid dehydrogenase [152]. In addition, *CYP3A4*, the prototypical PXR-target gene, is involved in the metabolism of steroid hormones. In fact, cortisol and

testosterone 6β-hydroxylase activities are often used as biomarkers for *CYP3A4* induction or inhibition [153, 154]. The CYP3A4 enzyme also catalyzes the 6β- and 16α-hydroxylation of progesterone and the 2-, 4-, and 16-hydroxylation of estradiol [155-157]. Furthermore, a transgenic mouse line expressing the *CYP3A4* transgene has been characterized with an impaired lactation phenotype associated with significantly reduced serum estradiol levels [158]. This may be of relevance in the treatment of pregnant or lactating women with PXR activators. However, further research is required to clarify the role of PXR in the homeostasis of steroid hormones.

2.3.4 Bile Acid Homeostasis

Bile, which is produced and secreted by hepatocytes, is essential for the elimination of excess cholesterol and for the digestion and absorption of dietary lipids. Bile secretion is also an important pathway for the elimination of hydrophobic endobiotic and xenobiotic metabolites. Bile acids, a major component of bile, are detergents that can be extremely toxic if their levels become elevated. Therefore, bile acid levels are tightly regulated by multiple NRs including HNF-4α, liver x receptor (LXR), farnesoid x receptor (FXR), CAR and PXR [118, 159-161]. While the physiological regulation of bile acid and cholesterol levels is primarily mediated by LXR and FXR by means of endogenous oxysterol and bile acid ligands, respectively, in the nanomolar range, PXR responds to the same ligands at micromolar concentrations in pathophysiological situations. The PXR protein is therefore involved in the regulation of the biosynthesis, transport and metabolism of cholesterol

and bile acid metabolites when levels of these endogenous compounds rise to excessive concentrations such as occurs during hypercholesterolemia and cholestasis.

Bile acids are produced in the liver by several enzyme dependent steps with the rate limiting step being the 7-hydroxylation of cholesterol by the CYP7A1 enzyme. In fact, it has been known for several years that the rodent PXR agonist PCN represses the activity of the Cyp7a1 enzyme [162]. Gene knockout studies confirmed that the repression of *Cyp7a1* gene expression by PCN is mediated by PXR (92, 118). Further studies suggest that PXR activation promotes PXR interaction with HNF-4α and blocks PPARγ co-activator-1α (PGC-1α) co-activation of HNF-4α, thereby resulting in inhibition of *Cyp7a1* gene transcription [163]. In addition to negatively regulating *CYP7A*, PXR activation positively regulates the expression of genes involved in bile acid metabolism and transport. The CYP3A4 enzyme catalyzes the hydroxylation of bile acids, and OatP2 and Mrp2 transport bile acids across the sinusoidal and canalicular membranes of hepatocytes.

During cholestasis, toxic levels (5-10μM) of lithocholic acid (LCA), a secondary bile acid, have been reported in the livers of patients [164]. Notably, at low micromolar concentrations certain bile acids and bile acid precursors such as LCA, cholic acid (CA) and chenodeoxycholic acid (CDCA) have been shown to activate PXR (92, 120). PXR activation then decreases bile acid synthesis via the down-regulation of *Cyp7a1* and accelerates bile acid metabolism through the up-regulation of metabolic enzymes and transporters. OatP2 is located on the sinusoidal membrane of the hepatocyte and is involved in the cellular uptake of bile acids.

Induction of OatP2 activity following PXR activation would increase the uptake of bile acids from the blood into the hepatocyte where the detoxification pathways such as hydroxylation and sulfation could take place by CYP3A4 and selective sulfotransferase enzymes (115, 118).

The effects of bile acids on their own synthesis are known to be mediated, in part, by small heterodimer partner (SHP), an NR-like protein that lacks a conventional DBD. FXR induces expression of the *Shp* gene, which in turn inhibits the transcription of the *Cyp7a1* gene [160]. SHP has also been shown to interact with PXR in a ligand-dependent manner and represses its activity [165]. However, SHP-null mice fail to repress *Cyp7a1* expression in response to specific FXR agonists, but repression of *Cyp7a1* is retained in SHP-null mice fed bile acids (166). This demonstrates the existence of a PXR-mediated repression pathway of bile acid signaling that is independent of SHP and will be discussed later in this chapter.

2.3.5 Cholesterol Toxicity

Cholesterol is a critical component of cell membranes and a precursor to steroid hormones. However, abnormally high levels of cholesterol contribute to several pathological conditions including coronary artery disease and atherosclerosis. In addition, oxysterols, which are oxidized metabolites of cholesterol, are toxic to cells [166]. The oxysterols 25-hydroxycholesterol and 24(S), 25-epoxycholesterol are able to induce *CYP3A* gene expression in rodent hepatocytes in a PXR-dependent manner (168). This suggests the possibility that drugs that are not direct PXR ligands

could cause drug-drug interactions by altering cholesterol metabolism that leads to the generation of oxysterols.

PXR may also be important in maintaining cholesterol homeostasis. There is an inverse relationship between levels of high density lipoprotein (HDL) cholesterol and the risk of coronary artery disease. PXR agonists elicited increases in serum HDL and serum apolipoprotein A1 (apoA1) levels in wild-type, but not PXR-null mice [167]. In addition, CA treatment produces significant decreases in HDL cholesterol and apoA1 in mice. However, PXR expression has been shown to antagonize this effect [168]. Finally, bile duct ligation experiments conducted in mice displayed increases in total serum cholesterol, which is attenuated by the deletion of PXR (171). Additional studies suggest that PXR regulates hepatic cholesterol transport by inhibiting genes central to cholesterol uptake and efflux. Specifically the ATP-binding cassette transporter A1 (ABCA1) and the scavenger receptor class B type 1 (SR-B1) which are both major factors in the exchange of cholesterol between cells and HDL [169].

Under normal conditions, dietary cholesterol does not cause toxicity due to complex homeostatic mechanisms. However, a recent study has shown that PXR-null mice are sensitive to diet-induced cholesterol toxicity. Feeding a diet that elicits the accumulation of cholesterol and its metabolites had no effect on the survival of wild-type mice. Yet, PXR-null mice showed acute lethality with signs of hepato-renal failure when fed the same diet [170]. This study suggests that the PXR signaling pathway protects the body from dietary cholesterol metabolites. Further investigation

is required to elucidate the protective mechanism, but this study raises an interesting notion that PXR ligands may alleviate cholestatic liver diseases and the associated hepato-renal failure.

2.3.6 Heme Homeostasis

Heme is an essential component of numerous hemoproteins with functions including oxygen transport, energy metabolism, and drug biotransformation. The liver is a major site of heme biosynthesis where CYPs rely on heme to catalyze the oxidation of endobiotic and xenobiotic compounds. However, the production of heme must be tightly regulated since accumulation of free heme within a cell leads to toxicity. The first and rate-limiting step in the heme biosynthesis pathway is 5-aminolevulinic acid synthase (ALAS). Studies on the regulation of *Alas* gene expression in mice have identified a drug-responsive enhancer element 17 kb upstream from the transcriptional start site of the gene. Reporter gene experiments indicated that activation of this response element could be mediated by PXR. *In vivo*, *Alas* gene expression is induced by prototypical PXR activators in wild-type, but not PXR-null mice [171]. Further studies identified an additional regulatory element located 20.1 kb upstream of the ALAS gene and gel shift assays demonstrated that the PXR protein is capable of binding to both of these regulatory response elements [172].

The ability of ALAS to respond to regulation by PXR suggests that PXR is involved in the control of the biosynthesis of heme. However, to date, the research completed in this area is limited. Understanding PXR-mediated regulation of *Alas*

gene expression could be valuable in the treatment of porphyria diseases, in which excess heme intermediates accumulate in tissues and lead to tissue damage and severe neurological problems.

2.3.7 Bilirubin Clearance

Bilirubin is the primary byproduct of heme protein catabolism. Accumulation of bilirubin results in hyperbilirubinemia and jaundice. Congenital hyperbilirubinemias are conditions in which there is an abnormal serum bilirubin level without abnormalities in liver function. The congenital hyperbilirubinemias are divided into two groups; conjugated (Dubin-johnson syndrome and Rotor syndrome) and unconjugated (Crigler-najjar syndrome and Gilbert's syndrome). Crigler-najjar and Gilbert's syndrome are caused by defects in the *UGT1A1* gene. Dubin-johnson syndrome is caused by a defect in the *MRP2* gene and the gene responsible for Rotor syndrome has not yet been identified [173, 174]. However, the causative genes in 3 of 4 congenital hyperbilirubinemias are regulated by PXR. Bilirubin is transported into hepatocytes by OatP2, where it is then glucuronidated by Ugt1a1 [175, 176]. The conjugated bilirubin is then transported by Mrp2 into the bile [177]. The role of PXR in bilirubin clearance was further confirmed with the use of VP-hPXR transgenic mice, in which the hPXR transgene is rendered constitutively active and ligand-independent by fusion with the potent transcriptional activator VP16. One hour after the mice were given a single dose of bilirubin, the remaining serum levels of both total and conjugated bilirubin in the transgenic mice were less than half of that observed in their wild-type littermates [121, 178]. Activation of PXR and up-

regulation of Ugt1a1 activity appears to be sufficient for conjugation and clearance of bilirubin in this mouse model. Since CAR is not expressed in neonates, PXR activation may be an interesting drug target for the treatment of neonatal or acquired forms of jaundice.

2.3.8 Vitamin D and Bone Mineral Homeostasis

Vitamin D promotes bone formation and is essential for skeleton development. Vitamin D deficiency leads to bone softening diseases such as rickets and osteomalacia. In addition, prolonged therapy with rifampicin has been shown to cause vitamin D deficiency or osteomalacia, especially in patients with low vitamin D stores [179, 180]. 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D_3$) is the active form of vitamin D in humans and is synthesized from vitamin D_3 by hepatic CYP27A1 and CYP2R1. $1,25(OH)_2D_3$ mediates its biological effect by binding to the VDR. VDR activation leads to the maintenance of calcium and phosphorus levels in the blood and to the maintenance of bone content [181]. CYP24 is well known to be the major enzyme that contributes to the metabolism of $1,25(OH)_2D_3$ to the inactive form 1,24,25-trihydroxyvitamin D_3 ($1,24,25(OH)_3D_3$). *CYP24* has been identified as a PXR-target gene by both *in vivo* and *in vitro* studies and PXR has been shown to bind to and transactivate the *CYP24* promoter [182]. This suggests that drugs that are PXR ligands can activate *CYP24* expression and alter $1,25(OH)_2D_3$ homeostasis. However, another study has suggested that the PXR-target gene *CYP3A4* is the major source of $1,25(OH)_2D_3$ metabolism in liver. Although the affinity and efficiency of $1,25(OH)_2D_3$ metabolism by the CYP3A4 enzyme is 10 fold lower than that of

CYP24 [183]. A follow up study showed that activation of PXR did not induce *CYP24* expression or transactivate the *CYP24* promoter [184]. In fact, PXR may repress vitamin D_3 activation of the *CYP24* gene by preventing the dissociation of the co-repressor silencing mediator for retinoid and thyroid hormone receptors (SMRT) from VDR on the *CYP24* promoter. The degree of PXR-mediated locking of SMRT appears to be dependent on the ratio of vitamin D to the PXR activator rifampicin [185]. Whatever the effect of the CYP24 and CYP3A4 enzymes on $1,25(OH)_2D_3$ homeostasis, the role of PXR in metabolic bone disorders remains unclear.

Vitamin K_2 is a critical nutrient required for blood clotting and plays a role in bone formation. In fact, vitamin K_2 supplementation increases bone density *in vivo* and is used clinically in the management of osteoporosis. Vitamin K_2 has been identified as a PXR ligand [150]. *In vitro*, vitamin K_2 was able to induce osteoblast bone markers in primary osteocytes isolated from wildtype, but not PXR-null mice [151]. Further studies indicate that the osteoblastgenic transcription factor MSX2 is a PXR-target gene and mediates the osteoprotective action of vitamin K_2 [125]. In this manner, PXR plays a novel role as a mediator in bone homeostasis and may be an effective drug target in the treatment of osteoporosis.

2.3.9 Inflammatory Response

Exposure to xenobiotics can impair immune function. In fact, it is a long-standing observation that rifampicin tends to suppress immunological responses in liver cells [186-188]. Recent publications have demonstrated a mutual inhibition between PXR and the inflammatory mediator nuclear transcription factor kappaB

(NF-κB), thus providing a potential molecular mechanism that links xenobiotic metabolism and inflammation [189, 190]. Activation of PXR by rifampicin suppresses the expression of typical NF-κB target-genes such as cyclooxygenase-2 (*COX-2*), tumor necrosis factor α (*TNFα*), intercellular adhesion molecule-1 (*ICAM-1*) and several interleukins (*IL*s) [190]. Conversely, NF-κB activation by lipopolysaccharide (LPS) and TNFα results in the suppression of CYP3A4 activity through interactions of NF-κB with the PXR-RXR complex [191]. Furthermore, hepatocytes derived from PXR-null mice have elevated NF-κB target-gene expression compared to hepatocytes from wild-type mice. The PXR-null mice also exhibit heightened signs of inflammation in their small bowel [190]. This could be due to the loss of negative regulation of NF-κB by PXR or due to inadequate clearance of toxic substances from this tissue.

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the digestive tract including Crohn's disease and ulcerative colitis. The etiology of IBD is unknown. However, PXR was identified as a gene strongly associated with the susceptibility to IBD [192]. In patients with IBD, decreased expression of PXR and PXR-target genes is observed [193, 194]. In the dextran sulfate sodium (DSS)-induced IBD mouse model, treatment with the PXR agonist PCN protected against DSS-induced colitis compared to vehicle-treated mice. However this treatment did not decrease the severity of DSS-induced colitis in PXR-null mice [195]. This indicates a role for PXR in protection against IBD. Interestingly, two drugs approved for the treatment IBD, budesonide and rifaximin, have recently been identified as

PXR activators [196, 197]. However, further human studies are required to assess the potential role of PXR activation in therapeutics for IBD.

2.3.10 Glucose Homeostasis

Glucose production is tightly controlled by insulin and glucagon signaling and plays a critical role in survival during fasting and starvation by regulating the transcription of genes that are involved in gluconeogenesis such as glucose-6-phosphatase (*G6Pase*) and phosphoenolpyruvate carboxykinase (*PEPCK*). Glucagon increases glucose production by up-regulating the transcription of these genes. Glucagon stimulates cAMP-activated protein kinase (PKA) to phosphorylate the cAMP response element binding protein (CREB), which then subsequently activates the transcription of *G6Pase* and *PEPCK* [198]. Insulin decreases glucose production by repressing the transcription of *G6Pase* and *PEPCK*. In the absence of insulin, FOXO1 activates the transcription of *G6Pase* and *PEPCK*. However, insulin signaling activates the phosphatidylinositol 3-kinase (PI3K)-Akt pathway to phosphorylate FOXO1, excluding it from the nucleus and resulting in the insulin-dependent repression of *G6Pase* and *PEPCK* [199, 200]. Previous observations have revealed functional links between insulin- and xenobiotic-mediated signaling pathways. For example, it is known that treatment with drugs that are known activators of PXR represses the expression of hepatic gluconeogenic enzymes [201-203]. In fact, treatment with the mouse PXR activator PCN decreased blood glucose levels in fasting wild type but not PXR-null mice [204]. The *G6Pase* and *PEPCK* genes are also down-regulated in transgenic mice expressing constitutively activated

PXR [123]. It is becoming increasingly clear that PXR activation represses the gluconeogenic pathway by interfering with transcription factors and co-factors that are involved in the regulation of *G6Pase* and *PEPCK* gene expression.

FOXO1 is a transcription factor that positively controls the expression of genes involved in gluconeogenesis. FOXO1 has been shown to interact with several NRs in a ligand-dependent or -independent manner and behaves as either a co-repressor or co-activator [205, 206]. FOXO1 was found to be a co-activator of PXR-mediated transcription. In contrast, PXR inactivated FOXO1 transcriptional activity by preventing its binding to its response element in target genes such as *G6Pase* and *PEPCK* [203]. This provides one mechanism whereby drug activation of PXR could interfere with the gluconeogenic program. It has also been proposed that PXR could inhibit the expression of gluconeogenic enzymes by interfering with the CREB pathway. PXR activation results in the repression of PKA/CREB-mediated activation of the *G6Pase* promoter in mice and in human hepatocarcinoma cell lines. This occurs by the ligand-dependent binding of PXR to CREB, which prevents CREB interaction with the cAMP response element (CRE) on the *G6Pase* promoter [207]. Finally, PGC-1α is a glucagon-activated gene that binds to and activates HNF-4α mediated transcription of *G6Pase* and *PEPCK*. Ligand-activated PXR dissociates PGC-1α from the HNF-4α complex through direct competition, thus repressing the transcription of *PEPCK* and *G6Pase* [202]. Since PGC-1α is also a co-factor for CREB- and FOXO1-mediated expression of gluconeogenic target-genes, a similar mechanism implicating the squelching of PGC-1α by PXR from those transcription

factors could be likely. The underlying mechanism of how PXR represses glucose production appears to be the direct binding of PXR to transcription factors and accessory proteins that activate gene expression critical for the gluconeogenic program such as FOXO1, CREB, and PGC-1α.

2.3.11 Lipid Metabolism

In addition to gluconeogenesis, hepatic lipid metabolism plays an important role in survival during periods of fasting and starvation. When blood glucose levels are low, the liver increases fatty acid oxidation and ketogenesis to provide extra-hepatic tissues with ketone bodies through β-oxidation and ketogenesis. The liver also decreases lipogenesis to lessen hepatic storage of triglycerides. Under these conditions, carnitine palmitoyltransferase 1A (CPT1A) and mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2), the key enzymes in β-oxidation and ketogenesis, are up-regulated [208, 209]. On the other hand, stearoyl-CoA desaturase 1 (SCD1), a key enzyme in the synthesis of unsaturated fatty acids is up-regulated by glucose [210]. In the absence of insulin, FOXA2 activates the transcription of *CPT1A* and *HMGCS2*. However, insulin signaling inactivates FOXA2 through the PI3K-Akt pathway resulting in the repression of *CPT1A* and *HMGCS2* [211]. Insulin also increases the transcription of *SCD1* by activating the lipogenic transcription factor sterol regulatory element-binding protein (SREBP) [212]. It is known that treatment with drugs, now classified as PXR activators, affect lipid metabolism in patients. For example, treatment with rifampicin or carbamazepine appears to induce

hepatic steatosis, characterized by the accumulation of triglycerides in liver [213, 214].

Recent studies have shown that treatment with PCN down-regulates the expression of *CPT1A* and *HMGCS2* in wild type, but not PXR-null mice. Activated PXR physically interacts with FOXA2 through their ligand and DNA binding domains, thereby preventing FOXA2 from binding to its response element in the *CPT1A* and *HMGCS2* promoters [204]. In addition, it has been shown that HNF-4α can activate *CPT1A* gene transcription [215]. As previously described, it has been demonstrated that PXR interferes with HNF-4α signaling by targeting PGC-1α and producing a squelching effect [202]. Since HNF-4α and PGC-1α are jointly involved in the regulation of *CPT1A*, it is likely that this type of crosstalk applies to this gene as well. Furthermore, the expression of activated PXR in transgenic mice resulted in increased hepatic lipid accumulation that was independent of SREBP, but associated with increased expression of the free fatty acid transporter CD36 and accessory lipgenic enzymes such as SCD1 and long chain free fatty acid elongase [123]. *CD36* transcription is activated by free fatty acids and by PPARγ [216]. However, PXR may promote hepatic steatosis by increasing the expression of CD36 both directly on the *CD36* promoter and indirectly through PXR-mediated activation of PPARγ [123, 217]. In addition, *Insig-1* has recently been identified as a novel PXR-target gene. Activation of *Insig-1* gene expression by drugs leads to reduced levels of SREBP and consequently to reduced target-gene expression of genes responsible for triglyceride synthesis [122]. A companion report showed that activation of SREBP by insulin

inhibits the transcriptional activity of PXR [218]. It appears that drug and lipid metabolism is interconnected within a complex network of transcriptional regulators including PXR. The role for PXR in the development of hepatic steatosis raises concern about the safety of drugs that are also PXR ligands. Overall, the role of PXR in lipid metabolism and steatosis warrants further investigation.

2.4 Mechanisms of PXR Activation

2.4.1 Ligand Binding

PXRs from all species examined can bind to a myriad of structurally diverse xenobiotic and endobiotic compounds. As previously described, the determination of the structure of the human PXR-LBD by X-ray crystallography has illuminated the molecular basis of PXR ligand-binding properties and has identified several residues that are critical for ligand binding. In addition, two types of ligand binding assays have been developed for PXR. The first is a direct scintillation proximity assay (SPA), which consists of genetically expressed and isolated receptors incubated with a test compound and a radiolabeled ligand. Competition of the radiolabeled ligand with the test compound is measured and an IC_{50} can be determined [46, 219]. A second approach that has been used to determine whether compounds bind to PXR is the co-activator receptor ligand assay (CARLA). The binding of an agonist to PXR results in a conformational change that permits interactions with the co-activator SRC-1. This interaction can be measured by co-precipitation of radiolabeled SRC-1 protein with PXR [42]. Studies using SPA and CARLA have demonstrated that many of the compounds that activate PXR do so by binding directly to the receptor. These

binding assays are fairly straightforward to conduct; however their simplicity can also be a deficit. For example, there is no cell membrane to act as a barrier to drug access. Cell based reporter gene assays represent a more complex assay to measure PXR activation by ligands and are often used to screen for PXR ligands in a high throughput format. The reporter gene assay is comprised of expression vectors for full length PXR and a variation of the *CYP3A* promoter coupled to a reporter gene. In this assay, increased luminescence or reporter gene activity is measured as an indication of *CYP3A* induction potential and an EC_{50} value can be determined. Typically there is a good correlation between EC_{50} and IC_{50} values in PXR transactivation and binding assays [220, 221].

In silico modeling of PXR ligand binding would have an important role in drug development, especially if such models could eliminate drug candidates that may activate PXR and cause drug-drug interactions early in the drug development process. However, the development of predictive *in silico* models of PXR-ligand interactions has proven to be very difficult. Three studies have utilized molecular modeling analysis for PXR ligands. The first study suggested that hydrophobicity of the ligand and adequate distance between the hydrogen-bond acceptor and the hydrophobic group is important for hPXR activation [222]. The second study utilized data on 12 diverse PXR activators to develop a three-dimensional pharmacophore. The pharmacophore was consistent with the hydrophobic nature of the PXR-LBD and was validated using a set of 28 known PXR ligands [223]. Finally, the third study suggests that highly active compounds share hydrophobic features that allow the

ligand to occupy large areas of the predominantly hydrophobic binding pocket [224]. So far, it is not possible to utilize the crystal structure of PXR for predictive *in silico* modeling. In fact, the broad specificity and the flexible nature of the PXR-LBD make structure based modeling very challenging.

2.4.2 DNA Binding

PXR binds as a heterodimer with RXRα to response elements composed of two copies of the NR binding motif AG(G/T)TCA in the promoter regions of genes [37]. The PXR-DBD is similar in structure to the RXRα-DBD, which is a double zinc-finger motif that binds to DNA in a sequence-specific manner. The response elements are arranged as direct repeats with 3 to 5 bases separating the DBD binding sites (DR-3, DR-4, and DR-5 elements), as well as everted repeats separated by 6 or 8 bases (ER-6 and ER-8 elements) (Figure 2-7) [42, 43, 117]. The PXR-RXR heterodimer was initially shown to bind to the DR-3 response elements in the *CYP3A23* and *CYP3A2* promoters [41, 94]. PXR binds to a DR-3 element and an ER-6 element in the promoter of its prototypical target-gene *CYP3A4* [42, 44, 94]. In addition, DR-3 and ER-6 response elements are conserved in other xenobiotic-inducible CYPs including *CYP3A7* [225]. PXR also binds to DR-4 and DR-5 response elements conserved in gene promoters such as *CYP2B6* and *MDR1* [116, 226, 227]. Finally, PXR has been shown to bind to an ER-8 response element in the promoter of the *MRP2* gene [117]. Since PXR and RXRα form only a single type of heterodimeric complex, the regions that connect the DBDs and LBDs must allow considerable flexibility to account for variations in response elements.

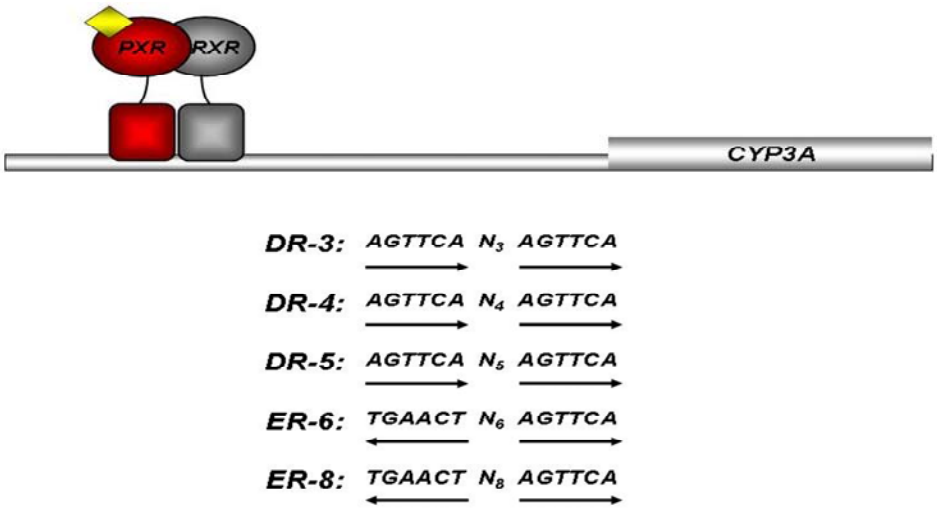

Figure 2-7. DNA-binding of PXR to its response elements. PXR binds as a heterodimer with RXRα to response elements composed of two copies of the NR binding motif half site AG(G/T)TCA. This consensus sequence is arranged either as a direct repeat with a 3-5 nucleotide spacer (DR-3, DR-4, and DR-5) or as an everted repeat with a 6 or 8 nucleotide spacer (ER-6 and ER-8).

2.4.3 Sub-cellular Localization

It was initially believed that PXR resides in the nucleus even in the absence of ligand. Previous reports based on immunocytochemistry and GFP-tagged PXR localization experiments indicated that hPXR localized exclusively to the nucleus, regardless of the presence or absence of ligand [74]. However, other groups have detected ligand-dependent translocation of PXR from the cytoplasm to the nucleus (Figure 2-8) [228, 229].

Nuclear translocation of PXR appears to be dependent on the presence of a nuclear localization signal (NLS) mapped to amino acid residues 66 to 92 within the C-terminal region of the DNA binding domain [228]. It was further demonstrated that PXR exists in the cytoplasm in a complex with heat shock protein 90 (HSP90) and cytoplasmic CAR retention protein (CCRP) in HepG2 cells. In fact, overexpression of CCRP increased the cytoplasmic level of the PXR, whereas a decrease in CCRP by treatment with siRNA repressed the PXR-mediated reporter activity in HepG2 cells [229]. It is apparent that CCRP and HSP90 are involved in the ligand-dependent nuclear translocation of PXR. However, the specific molecular mechanisms involved in this process are currently unknown.

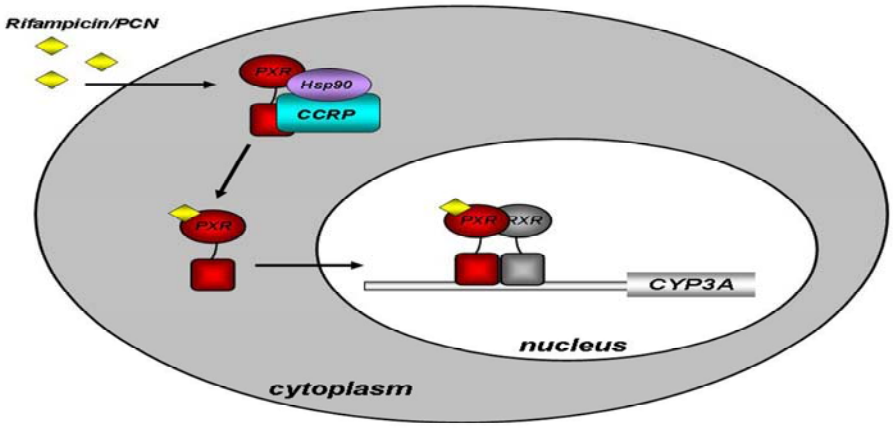

Figure 2-8. Ligand-dependent translocation of PXR from the cytoplasm to the nucleus. PXR exists in the cytoplasm in a complex with HSP90 and CCRP. Upon ligand binding PXR dissociates from CCRP and HSP90 and is translocated to the nucleus where it can activate transcription of its target-genes.

2.4.4 Co-factor Interactions

The full activity of PXR depends on its interaction with co-regulator proteins that do not bind to DNA directly but have a pronounced effect on gene expression [38]. In general terms, non-liganded PXR forms a complex with co-repressor proteins that inhibit transcriptional activity, often through the recruitment of other co-factor proteins that contain histone deacetylase (HDAC) activity. HDACs alter chromatin structure by promoting chromatin compaction, thus rendering enhancer regions of genes less accessible to the necessary basal transcriptional machinery. Activation of PXR by ligand-binding induces a conformational change which results in the dissociation of co-repressor protein complexes and the subsequent recruitment of co-activator proteins that enhance gene transcription, often through the recruitment of multi-protein complexes containing histone acetyltransferase (HAT) activity (Figure 2-9) [38]. However, this simplified model is complicated by the fact that receptor activity is also influenced by the nature of the ligand, promoter location and context, and the expression levels of specific protein cofactors in specific tissue types.

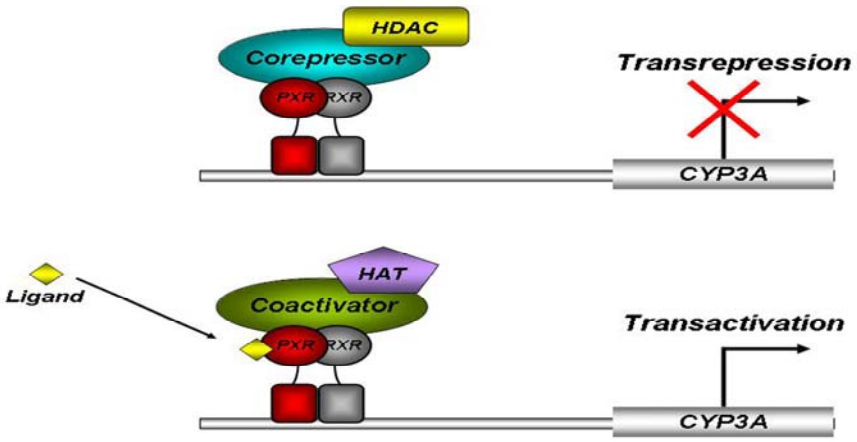

Figure 2-9. PXR activity is regulated by co-factor binding. PXR forms a complex with co-repressor proteins that inhibit transcriptional activity through the recruitment of HDACs. This promotes chromatin compaction and renders genes less accessible to basal transcriptional machinery. Ligand activation of PXR induces a conformational change, which results in the dissociation of co-repressor protein complexes and the subsequent recruitment of co-activaor complexes that enhance gene transcription through the recruitment of protein complexes HAT activity.

Most protein co-activators bind to PXR through the C-terminal AF-2 domain via an -LXXLL- motif. In the absence of ligand, AF-2 exists in a conformation that favors interactions with co-repressor proteins, whereas in the presence of ligand, AF-2 undergoes a conformational change that favors interactions with co-activator proteins [80]. The main co-activator protein involved in PXR activity is SRC-1, and the co-repressors involved include SMRT and nuclear receptor co-repressor (NCoR) proteins [230, 231]. Early studies determined via co-precipitation that PXR bound to SRC-1 [41]. Further studies confirmed that in the presence of ligand full length mouse PXR interacts with SRC-1 and the co-repressor receptor interacting protein 140 (RIP140) [99]. The species-specific nature of PXR activity emphasizes the importance of confirming these interactions with the human receptor. Several studies have confirmed human PXR's interaction with SRC-1 [89, 100, 104, 231]. One of these studies also demonstrated interactions between hPXR and additional co-activators including glucocorticoid receptor interacting protein 1 (GRIP1/SRC2), human activator for thyroid hormone and retinoid receptors (ACTR/SRC3), and PPAR binding protein (PBP) [104]. More recent studies have demonstrated that PXR binds to and is co-activated by PGC-1α on the *CYP2A6* promoter [232]. PGC-1α is unique in that it has been shown to co-activate NRs in a ligand-independent manner and could play a role in ligand-independent regulation of PXR activity.

The interaction of PXR with co-repressor proteins is not as well characterized as its interaction with co-activator proteins. SMRT and to a lesser extent NCoR interact with PXR in the absence of ligands, and different ligands have differing

abilities to prevent these interactions [104]. Several groups have examined co-repressor effects on the *CYP3A4* promoter in various species-specific cell lines. Transient transfection assays have shown that overexpression of SMRT inhibits PXR's transactivation of the *CYP3A4* promoter; whereas silencing of SMRT enhances the reporter expression in the human embryonic kidney cell line HEK293 [233]. In the human hepocarcinoma cell line HepG2, but not monkey kidney CV-1 cells, PXR showed increased interactions with SMRT in the presence of rifampicin, which lead to decreased PXR activity on the *CYP3A4* promoter [231]. In contrast, NCoR was responsible for the repression of ligand-induced *CYP3A4* expression in human colon carcinoma LS180 cells, but a separate mechanism is required for the repression of *MDR1* and *UGT1A1* in the same cell line [234]. It is clear that the modulation of PXR activity by protein co-factors is a highly complex ligand-, tissue-, signaling-, and promoter-specific process. Eulicidating the effects that co-regulator protein complexes have on the PXR transcriptional system remains an important area for future study.

2.4.5 Receptor Degradation

Degradation plays an important role in NR function by modulating protein levels. Relatively little is known about the degradation of PXR. However, preliminary studies show that PXR is degraded by the proteasome. PXR was found to interact with suppressor for gal-1 (SUG-1), a component of the 26S proteasome complex, in the presence of progesterone but not in the presence of endocrine disrupting chemicals [99]. A follow up study confirmed that PXR is degraded by the

proteasome and that ligands that do not enhance PXR's interaction with SUG-1, such as endocrine disrupting chemicals, block the degradation of PXR [235]. Therefore it is conceivable that ligands such as endocrine disrupting chemicals may affect PXR-mediated transcription of target genes in part through the up-regulation of the PXR protein level. The interaction between PXR and SUG-1 and the degradation of PXR by the proteasome appears to be complex and warrants further investigation.

2.5 Cell Signaling and PXR

2.5.1 Kinase Signaling and Post-translational Modification

Protein phosphorylation plays an important role in the regulation of NR function in general [236]. Drug-inducible CYP gene expression is known to be responsive to kinase signaling pathways; however, the exact mechanism by which these pathways intersect with PXR is unknown. Treatment of mouse hepatocytes with the PKA activator 8-Br-cAMP potentiated the induction of *Cyp3a11* by taxol and enhanced the binding of mouse PXR to SRC-1 and PBP. Further kinase assays show that PXR can serve as a substrate for PKA in vitro, suggesting one potential mechanism for PKA-mediated modulation of *CYP3A* gene expression [237]. It is also noteworthy that while PKA activation potentiates the drug-inducible expression of *Cyp3a11* in mouse hepatocytes, treatment of hepatocytes with 8-Br-cAMP serves as a repressive signal in both human and rat hepatocytes. Similar to the PXR ligand response, this suggests a species-specific effect for the modulation of drug-inducible *CYP3A* gene expression by PKA signaling. Activation of protein kinase C (PKC) signaling by phorbol myristate acetate (PMA) repressed PXR activity in reporter gene

assays and in hepatocytes by increasing the strength of interaction between PXR and NCoR, and by abolishing the ligand-dependent interaction between PXR and SRC-1. Interestingly, the protein phosphatase PP1/2A inhibitor okadaic acid strongly represses PXR-dependent transactivation [238]. In addition, cyclin-dependent kinase 2 (Cdk2) attenuated the activation of *CYP3A4* gene expression. PXR is a suitable substrate for the Cdk2 enzyme *in vitro*, and a phosphomimetic mutation at a putative Cdk phosphorylation site at (S350D) impaired the function of hPXR, whereas a phosphorylation-deficient mutation (S350A) conferred resistance to the repressive effects of Cdk2 on a reporter gene in HepG2 cells [239]. The results of these studies suggest that the activity of PXR is modulated by changes in phosphorylation within the cell, although direct phosphorylation of PXR was not demonstrated *in vivo*. PXR could be involved in integrating external signals via phosphorylation, but these observations need to be investigated further.

Post-translational modification of co-factors and PXR-interacting proteins may also have an impact on PXR transactivation capacity. For example, phosphorylation of RXRα at serine 32 inhibits the activity of several nuclear receptors, including PXR, that heterodimerize with RXRα [240]. The specificity and activity of NR co-factors is also regulated by kinase signaling and phosphorylation. Phosphorylation of SMRT on the C-terminal receptor interaction domain by the mitogen activated protein kinase (MAPK) kinase-kinase MEKK1 inhibits the interaction between SMRT and NRs [241]. In contrast, phosphorylation of SMRT by casein kinase 2 (CK2) on serine 1492 stabilizes SMRT-NR interactions [242]. In

contrast to SMRT, NCoR is refractory to MEKK1 phosphorylation and does not release NR partners in response to MEKK1 [243]. Since SMRT and NCoR exist in corepressor multiprotein complexes, their activity may be altered by kinase signaling that results in the phoshorylation of an HDAC. For example, phosphorylation of HDAC4 enhances its nuclear accumulation, whereas phosphorylation of HDAC1 and HDAC2 alters their interactions with corepressor proteins [244-246].

Seven phosphorylation sites for SRC-1 have been identified. Phosphorylation of SRC-1, SRC-2, and SRC-3 can be induced by a variety of environmental stimuli including epidermal growth factor (EGF), cAMP, cytokines, and steroid hormones [247-249]. In addition, the phosphorylation of SRCs induced by these agents is required for optimal coactivator activity. For example, SRC-1 phosphorylation at serines 1185 and 1179 is induced by cAMP and enhances the ligand-dependent and -independent activity of NRs. This phosphorylation event was also shown to be required for the interaction of SRC-1 with the HATs CREB binding protein (CBP) and p300/CBP associated factor (P/CAF) [247]. The p38 MAPK phosphorylates PGC-1α, a PXR-interacting coactivator protein, at three residues that occur in a region previously shown to interact with NRs [250]. However, it remains to be determined whether phosphorylation of PGC-1α affects PGC-1α's ability to interact with PXR. One study proposed that a repressor protein binds to the PGC-1α -LXXLL- motif, and that the interaction with NRs is terminated upon activation of p38 MAPK [251]. This suggests a model where a repressor protein and NRs compete to recruit PGC-1α and that activation of kinase signaling can shift that equilibrium. In

liver, insulin treatment resulted in the phosphorylation and inhibition of PGC-1α through Akt2/protein kinase B (PKB) [252]. Unraveling the detailed roles that phosphorylation of PXR and its coregulator proteins play in transcriptional control remains an important area for future study (Figure 2-10).

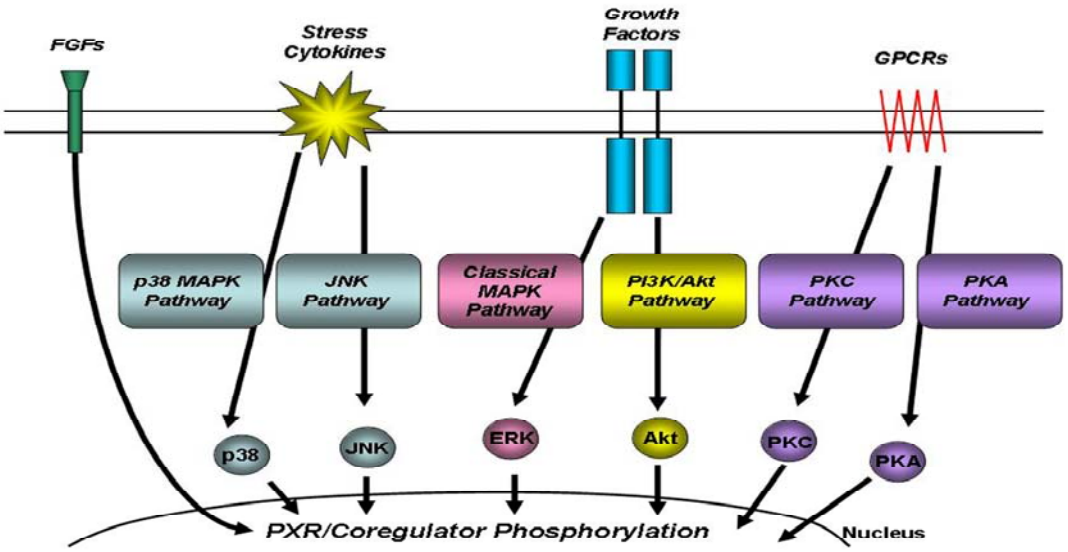

Figure 2-10. Activation of signaling pathways modulates PXR activity. Signaling pathways and phosphorylation events affect NRs and NR cofactors by disrupting protein-protein interactions, DNA-binding, subcellular localization, protein stability and transactivation capacity. The interface between signaling pathways and PXR warrants further study and is likely critical in the PXR response to environmental stimuli.

2.5.2 Cytokine Signaling

It has been known for a number of years that the constitutive and inducible expression of CYPs is modulated during inflammation and sepsis. Acute inflammation after surgery is associated with a decline in *CYP3A4* expression and activity and has an impact on the metabolism of prescription drugs [253]. In addition, gene expression profiling in patients afflicted with ulcerative colitis revealed disease- and tissue-specific decreases in the expression of PXR and its target genes [193]. In primary cultures of human hepatocytes, treatment with various proinflammatory cytokines down-regulates the basal and inducible expression of CYPs [254, 255]. In particular IL-6 has been shown to specifically inhibit both the basal expression of *CYP3A4* and its induction by rifampicin through PXR by at least 80%. This was associated with a reduction in the expression of *PXR* shortly after IL-6 treatment. Since the transcriptional activity of PXR was not affected by IL-6 in reporter gene assays, the loss of *CYP3A* expression and inducibility likely results from the negative regulation of *PXR* gene expression by IL-6 [256]. Interestingly, in the human intestinal Caco-2 cell line, treatment with proinflammatory cytokines decreased the mRNA expression of *CYP3A4* but increased the expression of *MDR1*, another PXR-target gene [257]. This finding suggests that inflammation and infection trigger several cellular responses that affect drug bioavailability. In this manner, cytokine signaling may have a promoter-specific effect on PXR activity, or may regulate these genes independently of PXR.

Similar responses to cytokine signaling have been observed in rodent hepatocyte and whole animal studies. In mice, the stimulation of inflammation by the injection of LPS reduced the expression *Cyp3a11* in liver. The decrease was associated with a marked reduction of *PXR* mRNA levels within 4 hours following treatment [258]. This effect appears to be PXR-dependent since the extent of PXR-target gene suppression by inflammation is significantly diminished in PXR-null mice [259]. Also noteworthy is that LPS induced the down-regulation of *PXR* and PXR-target genes in mouse placenta and fetal liver. The down-regulation of *PXR* and its target genes in placenta was attenuated by treatment with a free radical trapping agent, suggesting the possible involvement of reactive oxygen species (ROS) [260, 261]. In similar rat models of inflammation, inactivation of TNF4α prevented the down-regulation of the PXR-target gene *OatP2*, whereas inactivation of IL-1β prevented the down-regulation of the PXR-target gene *Mrp2* [262]. Finally, treatment of rat hepatocytes with interferon γ (IFNγ) reduced *CYP3A* mRNA expression as well as CYP3A protein levels and activity [263].

As described above, the activation of NF-κB by LPS and TNF4α results in the suppression of *CYP3A4* expression through interactions of NF-κB and the PXR-RXR complex [191]. This is one mechanism by which cytokine signaling can modulate PXR activity. Cytokine stimulation also activates PKC-dependent signaling pathways which are known to repress PXR activity [238]. Finally, it is clear that cytokine signaling can repress the expression of *PXR* itself, which results in a subsequent decrease in the expression of *CYP3A*. Taken together, the effect of

inflammation and sepsis on *PXR* expression and PXR-mediated responses is not well understood but remains an important area for future investigation.

2.5.3 Growth Factor Signaling

Some evidence is accumulating for the role of growth factors in regulating PXR signaling. Growth factors stimulate a variety of signaling cascades, are highly expressed upon liver damage and were shown to play a significant role in repair and regeneration [264]. Similar to the proinflammatory cytokines, some growth factors have been implicated in the down-regulation of *CYP* gene expression. For example, treatment of primary cultures of either human or rat hepatocytes with epidermal growth factor (EGF) down-regulated the constitutive and inducible expression of CYPs including *CYP1A*, *CYP3A*, *CYP2B*, and *CYP2C* [265-269]. Treatment of human hepatocytes with hepatocyte growth factor (HGF) similarly decreased the basal and inducible expression of *CYP3A4*, but had no effect on the expression of phase II UGT and GST enzymes [270]. *CYP3A4* expression is also modulated by a novel hepatotrophic growth factor, augmenter of liver regeneration (ALR). ALR has no effect on *PXR* expression but does repress rifampicin-induced expression of *CYP3A4* [271]. Another study aimed to investigate the effect that insulin-like growth factor-1 (IGF-1) and interferon (IFN)α treatment have on liver cirrhosis. Co-treatment of mice with IGF-1 and IFNα increased the expression of *PXR* and alleviated liver cirrhosis [272]. It is clear that growth factor signaling can modulate the expression of *CYP* genes, however the effect that growth factor signaling has on

PXR is unknown. It is possible that these growth factors activate signaling cascades that interface with PXR or other proteins associated with PXR activity.

Fibroblast growth factors (FGFs) function in processes such as development and wound healing. However, FGF19 (FGF15 in mouse) has recently emerged as a novel metabolic hormone. FGF19 has been identified as an FXR-target gene in liver and intestine. Gut-secreted FGF19 binds to its cell surface receptor FGF-receptor 4 (FGFR4) on hepatocytes and initiates a c-Jun N-terminal kinase (JNK) signaling pathway causing an inhibition of *CYP7A1* expression and subsequent bile acid synthesis [273, 274]. Recent studies have shown that FGF19 is also a PXR-target gene in intestine. LCA or rifampicin treatment leads to the induction of FGF19 promoter activity in intestinal cells [275]. This suggests a novel role for PXR in the LCA induced feedback-inhibition of bile acid synthesis via growth factor signaling.

2.6 PXR Crosstalk with Other Transcription Factors

The demonstration of crosstalk between PXR and other NR pathways indicates a more complex function for PXR. In general, the PXR signaling pathway comprises a linear series of events including (1) a stimulus, (2) heterodimerization, (3) protein cofactor binding, (4) a set of target genes, and (5) physiological functions carried out by gene products. However, this masks the fact that the PXR signaling pathway is interconnected with other pathways via multiple possibilities for crosstalk. These crosstalk events are apparently reciprocal, such that PXR activation and the expression of PXR-target genes are dependent on and affect the function of other

receptor pathways. The crosstalk between PXR and other transcription factors is summarized in Table 2-2.

Table 2-2. Crosstalk Between PXR and Other Transcription Factors. Crosstalk between PXR and other transcription pathways indicates a more complex function for PXR. This crosstalk is reciprocal and affects the physiological functions of both PXR and other pathways.

Crosstalk	Mechanism	Consequence
PXR-CAR	PXR and CAR share agonists; PXR and CAR share response elements within the same target genes	PXR and CAR control overlapping target genes; PXR and CAR are functionally redundant in regard to xenobiotic metabolism
PXR-FXR	FXR and PXR share agonists; FXR activates PXR target-genes; FXR controls *PXR* gene expression	Increases xenobiotic metabolism in response to bile acids; bile acid homeostasis is controlled by xenobiotics
PXR-LXR	LXR inhibits PXR transcriptional activity	Xenobiotic metabolism is decreased by oxysterols
PXR-SHP	SHP inhibits PXR transcriptional activity; PXR controls *SHP* expression	Xenobiotic metabolism is controlled by bile acids; bile acid homeostasis is controlled by xenobiotics
PXR-HNF-4α	PXR inhibits HNF-4α activity by competition for binding sites and PGC-1α	Gluconeogenesis and fatty acid homeostasis are altered by xenobiotics
PXR-VDR	PXR and VDR share response elements; CYP3A4 catabolizes vitamin D	Vitamin D controls xenobiotic metabolism; xenobiotics alter VDR target-gene expression and decrease active levels of vitamin D
PXR-PPARγ	PXR induces PPARγ expression; PXR induces PPARγ target gene *CD36*	PXR activation induces hepatic steatosis
PXR-PPARα	PPARα controls *PXR* gene expression	PPARα activation increases xenobiotic metabolism
PXR-GR	GR controls *PXR* gene expression	Glucocorticoids control xenobiotic metabolism
PXR-NF-κB	PXR and NF-κB are mutually repressive	Inflammation decreases xenobiotic metabolism; PXR activation alleviates inflammation
PXR-FOXO1	FOXO1 increases PXR transcriptional activity; PXR inhibits FOXO1 transcriptional activity	Reciprocal interaction between xenobiotic metabolism and gluconeogenesis
PXR-FOXA2	PXR inhibits FOXA2 transcriptional activity	Xenobiotics decrease fatty acid metabolism

2.6.1 PXR and CAR

Like PXR, CAR is a 'xeno-sensing' receptor that protects the body from an array of harmful chemicals by up-regulating the expression of drug-metabolizing enzymes. PXR and CAR share many of the same ligands and many genes have regulatory elements that can respond to both receptors. A diverse array of xenobiotics interacts with PXR and CAR as agonists, activators, or inverse agonists. PXR appears to be activated by more compounds when compared with CAR, but these receptors share certain ligands and target genes. For example, PB and 5β-pregnane-3, 20 dione activate both PXR and CAR, whereas clotrimazole and androstanol are activators of PXR, but inverse agonists of CAR. Similarly, bile acids, such as CA, are activators of PXR and suppressors of CAR transcriptional activity [47, 276]. In addition, since PXR and CAR regulate overlapping sets of target genes, the effect of these compounds on gene expression depends, to a certain extent, on the relative expression of both PXR and CAR. For example, guggulsterone, the active ingredient in guggulipid, is a PXR activator and a CAR inverse agonist [277, 278]. PXR-CAR crosstalk thus determines the activity of guggulsterone treatment toward expression of the prototypical CAR target-gene *CYP2B*. Mammalian two-hybrid reporter gene assays, demonstrated that treatment with guggulsterone favors the binding of the coactivator SRC-1 to PXR and displaces SRC-1 from CAR [278]. Therefore, the induction of *CYP2B* gene expression depends on the ratio of PXR to CAR.

PXR response elements are sometimes also recognized and transactivated by CAR and vice versa [117, 133, 227, 279, 280]. This would suggest an equal cross-regulation of target genes between PXR and CAR. However, even though PXR and CAR bind to and transactivate the same or similar response elements in the *CYP3A* and *CYP2B* promoters, their efficiencies in gene transcription are not the same. Human hepatocyte studies revealed nonselective induction of both *CYP2B6* and *CYP3A4* by PXR activation but marked preferential induction of *CYP2B6* by selective CAR activation [281]. Furthermore, the generation of PXR- and CAR-null mouse models was useful in determining that mPXR and mCAR regulate distinct and overlapping sets of target genes *in vivo* [110]. In addition to knockout mouse models, the development of ligands that are specific for either PXR or CAR, such as CITCO for CAR, may help in discriminating between CAR- and PXR-mediated gene expressions [282]. The response of drug-metabolizing enzymes to PXR and CAR activation may originate from either or both receptors depending on their relative abundance and their affinity for specific ligands and response elements. PXR and CAR are also competing for shared ligands and receptor interacting proteins including RXRα, SRC-1, and PGC-1α. It is likely that the crosstalk between PXR and CAR resulted from an adaptive advantage for organisms to increase their ability to detect and eliminate a wide variety of toxic compounds, a so-called 'metabolic safety net'.

2.6.2 PXR, FXR, LXR, and SHP

As mentioned above, bile acids are essential for cholesterol absorption and for the solubilization of dietary fats. The conversion of cholesterol to bile acids in the liver is initiated by the 7-hydroxylation of cholesterol by the CYP7A1 enzyme. Some bile acids are highly toxic, so their homeostasis is tightly controlled by a number of NRs including HNF4α, LXR, FXR, SHP, liver receptor homolog-1 (LRH-1), CAR and PXR [118, 159-161]. Oxysterols activate LXR to increase the expression of CYP7A1 [283]. Feedback repression of CYP7A1 is mediated by bile acids that activate FXR. The activation of FXR increases the expression of SHP, which inhibits positive regulators of CYP7A1 including HNF4α and LRH-1 [284, 285]. Bile acids are oxidized, conjugated, and transported by the products of PXR and CAR target-genes including CYP3A, UGTs, SULTs, MRPs, and OATP2 [286, 287]. Bile acid and xenobiotic detoxification pathways are therefore closely linked and many levels of crosstalk exist between the two.

FXR and PXR share some of the same ligands. LCA and its 3-keto derivative ursodeoxycholic acid are PXR agonists [92, 93]. Activation of PXR by LCA, which is highly toxic, results in the up-regulation of PXR-target genes including CYP3A4 and OATP2, which are involved in the metabolism and transport of bile acids [92, 118]. In addition, FXR controls *PXR* gene expression and can transactivate some PXR-target genes. Feeding wild-type mice with cholic acid or the FXR agonist GW4064 results in *PXR* and PXR-target gene induction, whereas no induction is observed in FXR knockout mice. FXR binds to a region in the *PXR* promoter and

results in increased transcription of *PXR* [288]. The findings that LCA induces expression of *CYP3A* in PXR-null mice and that FXR activates the *CYP3A4* promoter *in vitro* suggests that FXR may in involved in the regulation of PXR-target genes [93, 289]. In fact, two functional FXR response elements have been indentified in the xenobiotic responsive enhancer module (XREM) of *CYP3A4*, one of which is also known to bind to PXR [94]. Sequences known to be response elements of PXR have also been shown to be targets of FXR in the *SULT2A1* and *MRP2* genes [117, 290]. The combination of FXR-mediated activation of *PXR* and PXR-target genes provides an amplification mechanism for bile acid detoxification.

SHP represses CYP7A1 primarily by interacting with and inhibiting LRH-1. SHP has been shown to bind to and inhibit the activity of other transcription factors including CAR, LXR, FOXO1 and HNF4α [291-294]. SHP has also been shown to interact with PXR in a ligand-dependent manner and inhibits its transcriptional activity [165]. Furthermore, PXR may play a role in the regulation of *SHP* expression. *In silico* modeling identified a number of PXR response elements in the *SHP* promoter. PXR binds to these response elements *in vitro* and the induction of *SHP* expression by PXR in the presence of rifampicin was confirmed in HepG2 cells [295]. This finding combined with the fact that PXR is activated by bile acids suggests a mechanism for amplifying the detoxification response to bile acid toxicity by decreasing *CYP7A1* expression. However, it is interesting that the repressive effect of SHP on PXR should circumvent this effect. Nonetheless, the crosstalk

between SHP and PXR likely generates functional interference between bile acid homeostasis and xenobiotic detoxification pathways.

LXR enhances *CYP7A1* expression in response to cholesterol and oxysterols [283, 286]. Hydroxylated bile acids generated in part by CYP3A4 are LXR agonists. These compounds have also been shown to inhibit xenobiotic metabolism in liver. The LXR/RXR heterodimer can bind to the same response elements as PXR. In fact, reporter gene assays revealed that LXR inhibits the transcriptional activity of PXR through competition for binding sites [296]. This is consistent with previous observations that rats fed a high cholesterol diet have lower basal and inducible *CYP* expression compared to control animals [297]. Various levels of crosstalk reveal connections between the xenobiotic and bile acid pathways, suggesting that both pathways have established a long-standing cooperation throughout evolution.

2.6.3 PXR and HNF4α

HNF-4α is a transcription factor that binds to DNA as a homodimer and regulates the expression of several hepatic genes. Fetal and adult mice with a conditional deletion of HNF-4α have been used to determine the role of HNF-4α in *PXR* and PXR-target gene expression. The expression of *CYP3A* and *PXR* were suppressed by the inactivation of HNF-4α. In addition, elements in the *PXR* and *CYP3A* promoters were identified that confer with HNF-4α binding [60, 61]. These phenomena were confirmed in reporter gene assays of PXR-mediated transcription of *CYP3A4* in HepG2 cells [61]. In addition to *CYP3A4*, HNF-4α is also required for the maximal PXR-mediated induction of the *CYP2C9* promoter [298]. Recently a far

module in the *CYP3A4* promoter was shown to support the constitutive activity of *CYP3A4*. The far module, like a previously characterized distal module contains PXR response elements and elements recognized by HNF-4α. The presence of HNF-4α on the distal module increased rifampicin-induced reporter activity, but HNF-4α on the far module decreases it [299]. The difference between the far and distal modules in responding to HNF-4α suggests that HNF-4α plays a role in the fine-tuning of PXR-mediated regulation of *CYP3A4*.

PXR and HNF-4α also exhibit crosstalk through competition for co-factors. Ligand-activated PXR interferes with HNF-4α signaling by targeting the common coactivator PGC-1α. This squelching effect occurs through an increase in PXR-PGC-1α complex formation and an associated decrease in HNF-4α-PGC-1α complex formation, leading to a decrease in the expression of HNF-4α target genes involved in the regulation of bile acids and glucose such as *CYP7A1*, *G6Pase* and *PEPCK* [163, 202]. The crosstalk between PXR and HNF-4α plays a significant role in the regulation of xenobiotic, bile acid, and glucose homeostasis pathways. Therefore the application of drugs targeted to this crosstalk could be useful in the treatment of various pathological liver conditions.

2.6.4 PXR and VDR

VDR mediates the effects of $1,25(OH)_2D_3$ on a number of genes involved in biological functions such as bone mineralization. Several studies have demonstrated that $1,25(OH)_2D_3$ induces the expression of *CYP3A4*, *CYP2B6*, and *CYP2C9* in intestinal cell lines and human hepatocytes [300-302]. Since $1,25(OH)_2D_3$ does not

activate PXR, VDR was thought to be responsible for the observed induction. In fact, the VDR/RXR heterodimer binds to and transactivates PXR response elements in the *CYP3A4*, *CYP2B6* and *CYP2C9* genes [300, 302, 303]. In the absence of xenobiotic ligands, the basal expression of PXR-target genes may be controlled in part by VDR. Since vitamin D is present in our diets, it is possible that dietary habits may influence the inter-individual variability in the basal expression of PXR-target genes. Also similar to PXR, LCA binds to and transactivates VDR leading to increased expression of *CYP3A4* and *MRP3* [303]. It is suspected that in the presence of activators that PXR competes with VDR for response elements to enhance the transcription of target genes.

A reciprocal role in which PXR controls the expression of VDR-target genes has also been investigated. CYP24 is the major enzyme that contributes to the metabolism of $1,25(OH)_2D_3$ to the inactive form 1,24,25-trihydroxyvitamin D_3 ($1,24,25(OH)_3D_3$). In this manner CYP24 appears to regulate the negative feedback process that controls vitamin D homeostasis. *CYP24* has been identified as a PXR-target gene by both *in vivo* and *in vitro* studies and PXR has been shown to bind to and transactivate the *CYP24* promoter [182]. This suggests that PXR ligands may activate *CYP24* expression and alter $1,25(OH)_2D_3$ homeostasis. However, this had been called into question by a study that showed that activation of PXR did not induce *CYP24* expression or transactivate the *CYP24* promoter [184]. In fact, PXR may repress vitamin D_3-mediated activation of the *CYP24* gene by preventing the dissociation of SMRT from VDR on the *CYP24* promoter. The degree of PXR-

mediated squelching of SMRT appears to be dependent on the ratio of vitamin D to the PXR activator rifampicin [185].

An alternative mechanism for the role of PXR crosstalk with the vitamin D pathway has recently been proposed. The PXR-target gene *CYP3A4* may also be a major source of $1,25(OH)_2D_3$ metabolism in liver. Although the affinity and efficiency of $1,25(OH)_2D_3$ metabolism by CYP3A4 is 10 fold lower than that of CYP24, the relative expression of both CYPs suggest that CYP3A4 may play a dominant role in $1,25(OH)_2D_3$ metabolism in liver [183]. The effect of VDR-PXR crosstalk on the expression of *CYP24* and *CYP3A4* may play an important role in $1,25(OH)_2D_3$ homeostasis.

2.6.5 PXR and PPAR

PPARγ is a member of the PPAR family of NRs and is an important regulator of adipogenesis and lipid storage through the activation of target genes involved in lipid metabolism and transport. Activation of PXR results in increased hepatic lipid accumulation and is associated with increased expression of a number of genes involved in lipid metabolism including PPARγ, and the fatty acid transporter *CD36* [123]. The use of PXR transgenic and knockout mice showed that PXR is both necessary and sufficient for *CD36* gene activation. Additional promoter analyses revealed a PXR response element in the *CD36* promoter, establishing *CD36* as a direct transcriptional target of PXR [217]. Since PPARγ, a positive regulator of *CD36* expression, is a PXR-target gene, PXR may be able to regulate *CD36* expression directly or indirectly through its activation of *PPARγ.* This crosstalk

between PXR and PPARγ in the regulation of CD36 activity may play an important role in lipid homeostasis and the development of hepatic steatosis.

Additional evidence suggests that PPARα, another member of the PPAR family may play a role in the activation of the *PXR* gene itself. Several chemicals have been shown to regulate *PXR* mRNA expression including the PPARα agonists clofibrate and perfluorodecanoic acid [49]. Analysis of the *PXR* promoter has shown that PPARα-mediated induction of *PXR* expression occurs via a PPAR-binding site located approximately 1.3 kb upstream of the transcription start site. Ablation of this site prevented PPARα-mediated activation of *PXR* gene expression [304]. Therefore, compounds that increase *PXR* expression via PPARα signaling and compounds that interact with the PXR protein likely have synergistic effects on *CYP3A* induction. This crosstalk between PPARα and PXR represents another potential mechanism for drug interactions.

2.6.6 PXR, GR, and NF-κB

In primary cultures of human hepatocytes, expression of the *PXR* mRNA appears to be glucocorticoid-dependent [58, 305]. A functional glucocorticoid response element has not yet been identified in the *PXR* promoter region. However, any process that affects the expression or activity of GR is expected to affect the expression of *PXR*. In reporter gene assays, co-treatment of PXR ligands together with dexamethasone resulted in enhanced basal and ligand-dependent *CYP3A4* promoter activity. This induction was attenuated by treatment with a GR antagonist and by introduction of GR siRNA [306]. Ketoconazole and miconazole are known

antagonists of GR. Treatment of hepatocytes with these azole compounds down-regulates the expression of *PXR* and PXR-target genes [307]. Additional studies have shown that activated GR is involved synergistically in the xenobiotic-responsive regulation of PXR-target genes including *CYP2C8*, *CYP2B6*, *UGT1A1*, and *GSTA2* [113, 308-310].

It is well known that inflammation and sepsis are associated with the down-regulation of PXR-target genes. In fact, bacterial endotoxins and proinflammatory cytokines have been shown to reduce expression of *PXR* and PXR-target gene expression in human hepatocytes [256, 311]. These cytokines also activate NF-κB and this factor has been shown to bind and inactivate GR [312]. It has been shown that activation of NF-κB by IL-1β in human hepatocytes leads to the inhibition of GR activity, followed by the down-regulation of *PXR*, *CYP3A*, and *UGT1A1* [311]. Recent work has also demonstrated a mutual inhibition between PXR and NF-κB. Activation of PXR suppresses the expression of NF-κB target-genes, and PXR-null mice have elevated NF-κB target-gene expression compared to wild type mice [190]. Conversely, NF-κB activation inhibited PXR and its target genes through NF-κB-mediated disruption of the PXR-RXR complex [191]. During inflammation NF-κB activation may lead to the suppression of *PXR* expression and activity by both GR and NF-κB respectively. Nevertheless, the crosstalk between PXR and NF-κB represents the basis for the suppression of the activity of several hepatic CYPs during inflammation.

2.6.9 PXR, FOXO1, and FOXA2

FOXO transcription factors are involved in numerous biological processes including development, cell differentiation, apoptosis, gluconeogenesis, and lipid metabolism. Hepatic gluconeogenesis and lipid metabolism are tightly controlled by glucagon and insulin signaling. Gluconeogenesis is stimulated by glucocorticoids, cAMP and glucagon, and is negatively regulated by insulin and glucose. Two transcriptional regulators are known to be critical in the regulation of gluconeogenesis, FOXO1 and PGC-1α. PGC-1α expression up-regulates the gluconeogenic program and is a coactivator protein that associates with FOXO1 [313]. In the absence of insulin, FOXO1 activates the transcription of genes involved in gluconeogenesis. However, insulin signaling results in the phosphorylation of FOXO1, thereby excluding it from the nucleus. The net result is the insulin-dependent repression of genes involved in gluconeogenesis [199, 200]. The FOXO1 transcription factor binds to PXR and co-activates its transcriptional activity [203]. This protein-protein interaction may be of general significance for xenobiotic detoxification. In addition, the crosstalk between FOXO1 and PXR appears to be reciprocal. PXR inactivates FOXO1 transcriptional activity by preventing FOXO1 from binding to its response elements in gluconeogenic target genes [203]. Therefore, drug metabolism and gluconeogenesis may be co-regulated in response to insulin and/or xenobiotics. This data is consistent with the long-standing observation that diabetes enhances hepatic drug metabolism and that drugs that activate PXR suppress the expression of genes involved in gluconeogenesis [123, 201]. Another

possibility is that the objective of the crosstalk between FOXO1 and PXR is NADPH homeostasis. NADPH is essential for CYP activity. In liver, the pentose phosphate pathway generates NADPH through the conversion of glucose 6-phosphate to ribose 5-phosphate by glucose 6-phosphate dehydrogenase (G6PDH). In gluconeogenesis, glucose 6-phosphate is converted to glucose by G6Pase. The repression of gluconeogenesis by xenobiotic-activated PXR might be essential to maintain adequate levels of NADPH for xenobiotic detoxification. On the other hand, the decrease in PXR activity by insulin signaling is consistent with decreased NADPH production through the repression of gluconeogenesis.

When blood glucose is low, the liver metabolizes fatty acids via ketogenesis and β-oxidation to provide ketone bodies to extra-hepatic tissues. FOXA2 has been shown to positively regulate this process by controlling the transcription of target genes including *CPT1A* and *HMGCS2* [211, 314]. Treatment with PCN down-regulates the expression of *CPT1A* and *HMGCS2* in wild type, but not in PXR-null mice. It was further shown that activated PXR and FOXA2 physically interact through their ligand- and DNA-binding domains, respectively. This interaction prevents FOXA2 from binding to its response elements and leads to the repression of *CPT1A* and *HMGCS2* [204]. This crosstalk suggests that activated PXR repressed hepatic energy metabolism by decreasing both ketogenesis and β-oxidation.

2.7 Pre-clinical Modeling and Prediction of PXR Activity

2.7.1 Human Hepatocytes

Given the prominent role of PXR in regulating drug metabolism and drug elimination, assessing PXR activation is an important part of the drug development process. A range of screening models have been developed for the assessment of PXR activation potential in response to existing drugs and lead compounds. Aside from *in vivo* studies, short-term human hepatocyte cultures are the 'gold standard' for *in vitro* analysis of drug-mediated induction of enzymes and transporters. Some of the limitations of primary cultures of human hepatocytes are the cost, scarcity of supply, and high degrees of inter-individual variability that exists [315-317]. The development of immortalized human hepatocytes that maintain robust xenobiotic responsiveness may address this problem. However, due to these limitations, the use of high-throughput *in vitro* PXR activation and binding assays are commonly used to identify PXR activators. *In silico* modeling of PXR ligand binding would be a useful tool in drug development; however, as mentioned above, pharmacophore modeling of PXR ligand-binding is in the early stages of development.

2.7.2 In Vitro Activity Assays

A number of *in vitro* models are commonly used to study ligand interactions with PXR including SPA and CARLA as described previously. The most common approach is to use either transient or stably transfected cell-based reporter gene assays incorporating expression vectors for PXR and the XREM derived from the *CYP3A4* promoter linked to a reporter gene in human liver and intestinal cell lines. The

receptor expression plasmid can encode either the full-length PXR or a chimera between the PXR-LBD and the DBD of another heterologous transcription factor, such as the yeast transcription factor Gal4. The reporter gene plasmid contains binding sites for either full-length PXR or the PXR chimera upstream of a gene encoding an easily quantifiable reporter protein such as luciferase (Figure 2-11).

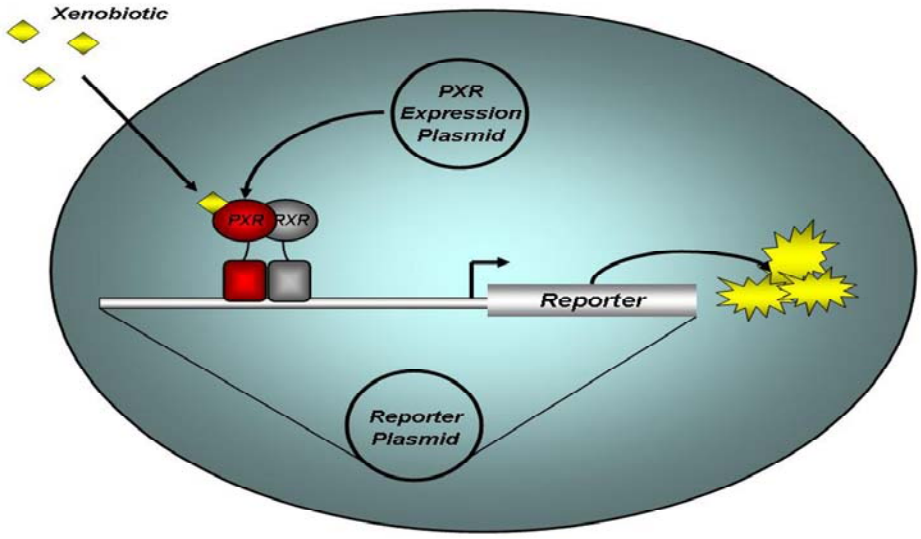

Figure 2-11. Cell-based reporter gene assay. Reporter gene assays are a common way to assess PXR activity. Host cells are transfected with a PXR expression plasmid and a reporter plasmid encoding an easily quantifiable reporter protein such as luciferase. PXR is expressed in cells, heterodimerized with endogenous RXR, and binds to specific response elements with in the reporter gene. The presence of a PXR ligand increases expression of the reporter gene, which can be detected using standard assays.

The advantages of reporter assays are the ability to specifically assess PXR activation without the contribution of other receptors. Although when using full-length PXR one must be aware that the cell line used may express endogenous PXR or other receptors that are capable of transactivating the reporter gene. The chimera system eliminates the background caused by endogenous receptors and permits ligand screening without considering the DNA-binding characteristics of PXR [316]. In order to account for transfection variability, stable cell lines containing both integral genes encoding human PXR and a reporter gene driven by XREM have been constructed. Results generated using the stable cell line strategies are similar to those generated from transient transfection analysis [318].

HepG2 is a commonly used cell line in reporter gene studies, but does have limitations compared to human hepatocytes. For example, the ability of compounds to induce *CYP* expression in human hepatocytes is not robustly reproduced in HepG2 [319]. There can also be inter-laboratory variability in the phenotype of HepG2 based on repeated passaging of the cell line and variable cell culture conditions. Another issue is that many experiments involve transfecting a receptor from one species into a cell line derived from another species. Given the species-specific nature of the PXR response, great care should be taken in the interpretation of such data. The cell lines currently available can not fully reproduce the hepatocyte, leading to discrepancies between the two systems. For example, St. John's wort is a more potent activator of PXR in reporter gene assays when compared with rifampicin, but the opposite is true when observing the induction of *CYP3A4* in human hepatocytes [320, 321].

Hepatocytes or in intact livers may be able to metabolize the compound studied and remove the compound from the cell via transporter proteins. Therefore, while reporter gene assays are useful tools for high throughput screening, positive results should be confirmed *in vivo* or in human hepatocytes.

2.7.3 PXR-null Mouse Models

It is difficult to extrapolate *in vitro* results to a clinical situation *in vivo* and cell lines are limited in the study of overall PXR function. As a result, PXR animal models are necessary to study PXR function in a whole animal system. Two PXR-null mouse models have been successfully generated using similar strategies of disrupting the mouse *Pxr* gene by homologous recombination [92, 322]. PXR-null mice have no overt phenotype and biochemical analysis showed no difference in serum cholesterol, triglycerides, glucose, or liver enzyme levels compared to wild type controls. These mice also develop and reproduce normally [134]. However, as expected, PXR-null mice do not respond to PXR ligands. PCN strongly induces PXR-target genes in wild type mice, but not in PXR-null animals [92, 322]. This was also observed at the level of enzyme activity in that PCN did not increase testosterone 6β-hydroxylation, nor did it decrease paralysis time by the muscle relaxant zoxazolamine, both measures of CYP3A activity, in PXR-null mice [92]. Consequently, the PXR-null mouse was validated as a reliable model to study PXR-dependent signaling pathways. It is also noteworthy that the basal level of *CYP3A* expression is increased roughly 3-4 fold in PXR-null mice compared to wild type controls [92]. Perhaps the absence of PXR allows other constitutively active

transcription factors such as CAR to transactivate the *CYP3A* promoter. Another possibility is that in the absence of ligand, PXR interacts with corepressor proteins on the *CYP3A* promoter and actively represses the transcription of that gene.

2.7.4 Humanized PXR Mouse Models

The major reason for developing a humanized PXR mouse model is the distinct species-specific difference in the response to PXR ligands. Toward this end, several humanized PXR mouse models have been successfully generated. Alb-hPXR and TTR-hPXR transgenic mouse models have been generated by the use of a cDNA fused to the liver-specific albumin and transthyretin promoters. These models have been developed in a PXR-null mouse background. As expected, these humanized PXR mice responded to the human-specific PXR activator rifampicin and showed little response to the mouse-specific PXR activator PCN (Figure 2-12) [101, 322].

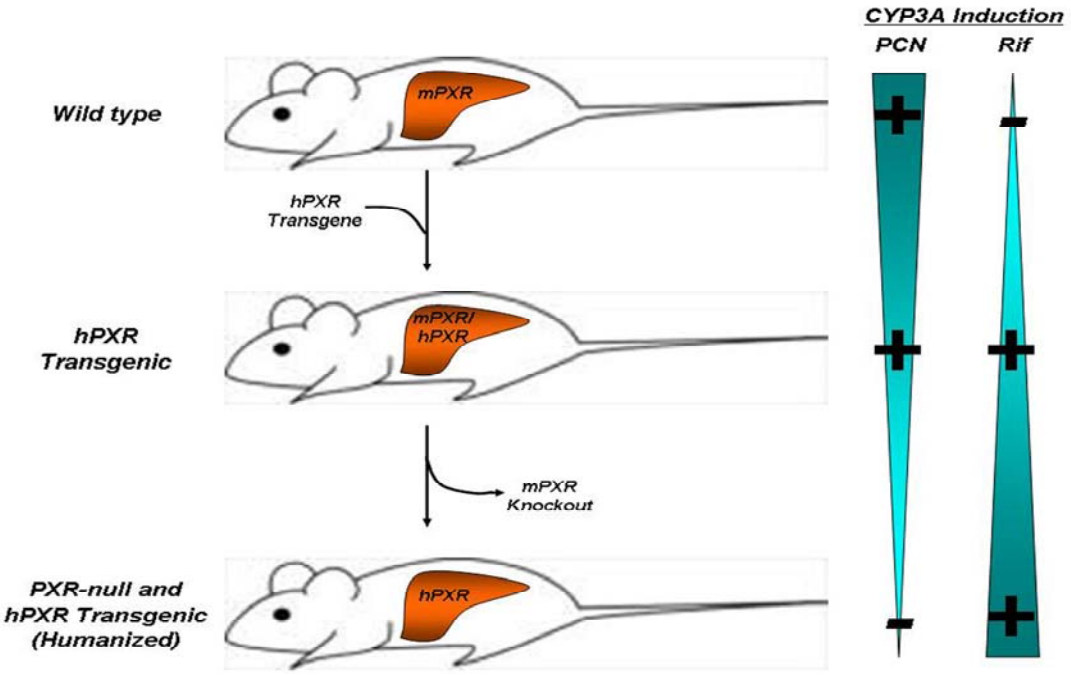

Figure 2-12. The development of humanized PXR mouse models. Several humanized PXR mouse models have been generated by the use of a hPXR cDNA fused to a liver-specific promoter. These models have been developed in a PXR-null mouse background. Humanized PXR mice respond to the hPXR activator rifampicin (Rif) and show little response to the mPXR activator PCN. Such models are powerful tools to study the effect of hPXR activation in a whole animal system.

These two mouse lines were generated using cDNA containing strong promoters to drive high expression of PXR in liver. Therefore, results obtained using these models do not directly recapitulate the expression pattern of human PXR in these mice, which lack expression of human PXR in extra-hepatic tissues. To address this issue, two functional BAC-hPXR models have been generated using a bacterial artificial chromosome (BAC) [323, 324]. The BAC transgene contains the complete *PXR* gene and is under control of the native hPXR promoter. These BAC-hPXR models represent a useful approach to address the effects of hPXR on drug metabolism and pharmacokinetics, especially since in humans PXR is also expressed in the gut where it is involved in xenobiotic metabolism and transport. A fourth model in which hPXR was fused to the coactivator VP16 was generated and produced a consitutively active hPXR mouse. The Alb-VP-hPXR mouse exhibits constitutive activation of PXR-target genes, as well as hepatomegaly, liver toxicity, and growth retardation compared to Alb-hPXR controls [322]. These phenotypes suggest that the sustained activation of PXR may be harmful. Although these humanized mice don't reflect human xenobiotic detoxification pathways with complete accuracy, they are powerful tools to study the effects of human PXR on xenobiotic detoxification in a whole animal system.

2.8 Therapeutic Opportunities

2.8.1 Hepatic Cholestasis

Cholestatic liver disease is characterized by the impairment of bile flow and the accumulation of bile acids and bilirubin. Adaptive regulation of phase I and II

metabolism and of the biliary transport system can minimize cholestatic liver injury. PXR activation decreases bile acid synthesis through down-regulation of *CYP7A1* and accelerates bile acid metabolism and elimination through the up-regulation of metabolic enzymes and transporters [118, 163]. Clinically, ligands for PXR such as rifampicin have long been used for the treatment of jaundice and pruritus associated with cholestatis. Rifampicin alleviated pruritus and reduced the serum concentrations of total and conjugated bile acids [325-328]. Rifampicin treatment led to increased expression of *MRP2* in patients with gall stones [329]. Furthermore, induction of *MRP2* expression along with increased glucuronidation of bilirubin by PXR-induced UGT1A1 enhances bilirubin detoxification [330]. However, the clinical data are controversial in regard to rifampicin treatment for cholestasis. In patients with or without cholestasis, rifampicin has been shown to increase plasma levels of bile acids within two hours of treatment [331]. Cholestatic hepatitis has been reported in humans treated with rifampicin [332]. In addition, a high incidence of hepatotoxicity has been reported in patients with biliary cirrhosis undergoing treatment with rifampicin [333, 334].

While many rodent studies have shown that LCA-induced liver damage can be alleviated by PXR activation, less is known about whether these strategies can be applied to other models of cholestasis. Administration of PXR ligands to mice with bile duct ligation reduced serum bile acid levels and increased bile acid clearance. Despite the improvement in cholestasis, markers of liver injury were increased in this study, possibly caused by the accumulation of PXR agonists in models of biliary

obstruction [126]. PXR is a promising target for the treatment of cholestasis. However, the risk of rifampicin-induced hepatotoxicity should be taken into account for patients with cholestasis and future studies are required to assess the safety of these treatments.

2.8.2 Hepatic Steatosis

Nonalcoholic fatty liver disease is the most common liver disorder in affluent societies and is characterized by the abnormal retention of lipids within the cells. Hepatic steatosis patients have few symptoms; however they are at increased risk of liver fibrosis, cirrhosis and cancer. In humans, steatosis is most often caused by either alcohol abuse or metabolic syndrome, but may also be induced by certain drugs or toxins. As previously discussed, PXR plays a role in lipid metabolism in the liver and its function may affect the pathogenesis of steatosis. Hepatic lipid accumulation was noted in humanized PXR mice treated with rifampicin [123]. It was recently shown that PXR may promote hepatic steatosis by increasing the expression of *CD36* either directly or indirectly through PXR-mediated activation of *PPARγ* [217].

The role of PXR in hepatic steatosis raises concern about the safety of drugs that are also PXR ligands. In humans, the PXR activators rifampicin and carbamazepine have been known to induce events of steatosis [213, 214]. Nevertheless, the revelation of the role of PXR in hepatic steatosis opens debate on whether these regulatory pathways can be therapeutically targeted in steatosis. If activation of *CD36* expression is causative in steatosis, then one can speculate that the

inhibition of PXR or the direct inhibition of CD36 activity may represent a novel strategy in steatosis treatment.

2.8.3 Inflammatory Bowel Disease

Inflammatory bowel disease (IBD) is a chronic inflammatory condition of the digestive tract occurring as ulcerative colitis or Crohn's disease. Ulcerative colitis is limited to the colon whereas Crohn's disease most commonly affects the small intestine, but could involve any part or the gastrointestinal tract. In the past, genetic, psychological, infectious, and immunological factors have all been implicated in the etiology of IBD. A recent report suggests that reduced expression and/or functional polymorphisms of PXR are associated with IBD [192]. In fact, decreased expression of *PXR* and PXR-target genes has been noted in patients with IBD [193, 194]. Progress is being made in the development of anti-inflammatory and immunosuppressive medications for the treatment of IBD. The identification for the role of PXR in inflammation and IBD may provide a new drug target for these conditions. Notably, budesonide, an anti-inflammatory drug frequently used in the treatment of IBD, has been recently identified as a PXR ligand [197]. In addition rifaximin, which was initially approved for the treatment of travelers' diarrhea, was found to be useful in the treatment of inflammatory gastrointestinal disorders. Rifaximin has been shown to be beneficial in the treatment of ulcerative colitis and moderate Crohn's disease as well as in the prevention of postoperative recurrence of IBD [335-337]. Furthermore, by using PXR-null and –humanized mouse models, rifaximin was identified as a gut-specific hPXR activator [196]. PXR appears to be a

promising target in the treatment of IBD; however, further studies are required to assess the potential role of PXR activation in such therapeutics.

2.8.4 Cancer and Chemotherapy

PXR plays an obvious role in cancer treatment because it is activated by a variety of common chemotherapeutic compounds like paclitaxel and cisplatin [104, 135]. The up-regulation of PXR-target genes involved in increased drug clearance is one of the reasons that such high doses of antineoplastic agents are required for clinical efficacy. It would be advantageous to identify compounds that limit PXR activation to avoid potential toxicities and drug interactions associated with chemotherapy. In addition, acquired resistance to chemotherapeutic agents is a major clinical problem and cause of failure in the treatment of cancer. Several targets have been shown to be related to chemo-resistance including efflux transporters, phase I and phase II enzymes, and DNA repair enzymes. Many of these targets are encoded by PXR-target genes such as *Mdr1*/p-glycoprotein, *MRP*s, *CYP3A*, *UGT*s, and *GST*s [338]. Activation of PXR induces the expression of these genes to accelerate the metabolism and elimination of chemotherapeutic agents, which may contribute to acquired drug resistance and multi-drug resistance.

A more basic link between PXR and the development of cancer is also emerging. Increased expression of PXR and its target-genes has been detected in cancerous tissues including breast, prostate, ovary, endometrium and colon [54, 339-342]. PXR also appears to be manipulated in cancer cells to promote tumor growth. A pattern of PXR up-regulation combined with estrogen receptor (ER) down-

regulation was identified in endometrial and breast cancer cells suggesting that PXR provides a growth advantage to neoplastic cells by processing steroid like compounds and xenobiotics [54, 341]. Breast cancer cells also have increased expression of PXR-target genes such as *OATP1A2* that is capable of mediating the cellular uptake of estrogen [56, 343]. In addition an anti-apoptotic role of PXR has been reported in human colon cancer cells [344].

2.8.5 Antifibrogenesis

PXR has recently been proposed as a target for anti-fibrotic therapy. In rats treated with carbon tetrachloride, liver necrosis and fibrogenesis are produced. PCN treatment has been shown to inhibit the extent of fibrosis in liver in a PXR-dependent manner [345]. In human hepatic stellate cells short-term treatment with rifampicin inhibited the expression of fibrosis related genes. Long-term treatment with rifampicin reduced the proliferation and trans-differentiation of hepatic stellate cells. All of the rifampicin-mediated effects in these cells were PXR-dependent [346]. The mechanism by which PXR alleviates fibrosis is unknown, but PXR may be a potential target for anti-fibrotic therapy.

2.8.6 Therapeutic Obstacles

PXR is an attractive target for drug discovery since its activity is regulated by small lipophilic molecules (Figure 2-13). However, the modulation of PXR activity leads to changes in the expression of multiple target-genes that are involved in multiple physiological processes. Therefore, therapeutic targeting of PXR may be associated with deleterious side effects. One opportunity in the development of drugs

that target PXR is to take advantage of selective receptor modulators (SRMs). SRMs are NR ligands that exhibit agonistic or antagonistic activity in a cell- or tissue-dependent manner. The classic SRM is tamoxifen, which can selectively activate or inhibit ERs and is commonly used in the treatment of breast cancer. Tamoxifen exhibits antagonist activity in breast and partial agonist activity in endometrium [347]. The expression profile of coregulator proteins and signaling pathways within different cell types likely contributes to the differential activities of SRMs. A study addressing the effect of various PXR ligands and potential promoter selectivity revealed that steroidal compounds preferentially induced PXR activity towards the *CYP3A* promoter when compared with the *MDR1* promoter. Conversely, anti-cancer agents preferentially induced the *MDR1* promoter when compared with the *CYP3A* promoter. The mechanism for this differential promoter activation was traced to the differential recruitment of co-activator proteins [135]. These results indicate that opportunities exist for the identification of selective PXR agonists that may be useful in the direct treatment of disease or in fine-tuning the efficacy of other medications. In order for PXR to be an effective therapeutic target, the activation of a potential therapeutic target gene must be separated from the activation of other genes involved in drug metabolism. A better understanding of coregulator proteins, signaling pathways, and receptor crosstalk that interface with PXR activity may provide alternative drug therapies toward that end.

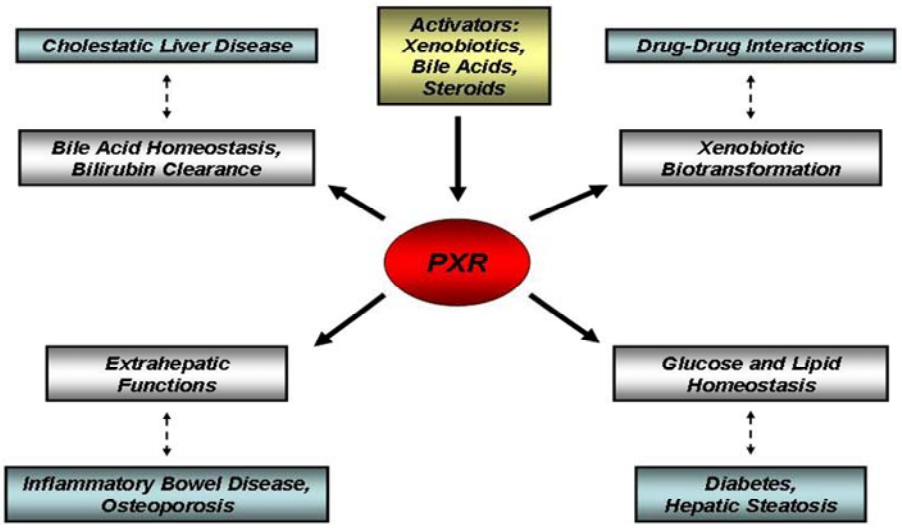

Figure 2-13. Physiological roles of PXR and their relation to disease states. PXR was originally characterized for its role in xenobiotic and endobiotic detoxification. However, recent evidence has described a role for PXR in glucose and lipid homeostasis, inflammation, and bone mineralization, to name a few. Further studies might reveal that PXR is a good potential drug target for the treatment of various diseases.

2.9 Conclusion

For decades it has been recognized that drug-inducible hepatic gene expression has a profound impact on xenobiotic biotransformation. Since the identification and molecular cloning of PXR in 1998, this fascinating receptor has been well characterized and is now recognized to play a major role in the transport, metabolism and clearance of xenobiotics and clinically prescribed drugs. The PXR protein is mainly expressed in liver and intestine where it is activated by a broad range of lipophilic compounds in a species-specific manner. Upon activation, the majority of the cellular PXR translocates to the nuclear compartment where it binds to specific PXR-response elements and increases the expression of numerous target genes involved in the xenobiotic biotransformation process. Ligand binding also alters the association of the PXR protein with co-regulator multi-protein complexes that bind to PXR and either enhance or repress transcription. In this manner PXR has a hepato-protective role and represents the basis for an important class of drug-drug interactions. PXR was originally characterized as a regulator of the homeostatic control of steroids, bile acid, and xenobiotics. However recent evidence has revealed a role for PXR in gluconeogenesis, lipid metabolism, and inflammation through either direct regulation or crosstalk with other transcription factors. Ligand binding is the primary mode of PXR activation, but several signaling pathways also interface with PXR and affect its overall responsiveness to environmental stimuli, likely by altering the phosphorylation status of PXR or its associated protein co-factors. Finally,

pharmacological manipulation of the complex network of factors that contribute to PXR activity present therapeutic opportunities in the treatment of numerous diseases.

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Chapter 3: The Traditional Chinese Herbal Remedy Tian Xian Activates PXR and Induces CYP3A Gene Expression in Hepatocytes

3.1 Introduction

Nuclear receptors comprise a large superfamily of transcription factors that are characterized by a conserved N-terminal zinc-finger type DNA-binding domain and a carboxy-terminal ligand-binding domain. They are involved in a variety of physiological, developmental, and toxicological processes [1]. Pregnane X receptor (PXR, NR1I2) was first cloned in 1998 by a research group at GlaxoWellcome as a part of an effort to identify new members of the nuclear receptor superfamily based upon homology and the mouse genome sequencing project [2].

Since then, PXR has been identified in various species, including human, monkey, cow, pig, rabbit, rat, mouse, chicken, fish, and worms [3-5]. In mammals, PXR is highly expressed in the major organs that are important in xenobiotic-biotransformation including the liver and intestine [2]. Numerous studies show that activation of PXR in the liver and intestine produces increased expression of a group of genes that encode proteins involved in the uptake, metabolism, and elimination of potentially toxic compounds [6-10].

It is well-established that PXR is a key regulator of xenobiotic-inducible CYP3A gene expression [11, 12]. In addition, PXR regulates the inducible expression of other genes involved in the metabolism of xenobiotic compounds such as CYP2B, CYP2C, CYP24, glutathione S-transferases, sulfotransferases, and glucuronosyltransferases [6, 13-16]. In rodents, PXR also regulates the expression of

genes encoding the drug transporter genes organic anion transporting polypeptide 1A4, P-glycoprotein/Mdr1, multi-drug resistance-associated protein 2, and multi-drug resistance-associated protein 3 [10, 17, 18]. Therefore, PXR activation has a complex nature. While it protects cells from toxic insults, it also represents the molecular basis for an important class of drug-drug interactions.

For example, if one drug activates PXR, it can be predicted that administration of this drug will promote the elimination of other co-administrated drugs that are also metabolized and eliminated by PXR-target gene products, thereby reducing the efficacy of many drug therapies in patients on combination therapy. Additionally, if one drug is a administered as a pro-drug, as is the case with certain anti-cancer therapeutic agents, and a PXR agonist is then co-administered, the resulting increased biotransformation of the pro-drug would likely produce profound and unwanted toxic side effects. This phenomenon is also observed with numerous herbal remedies including St. John's Wort, coleus forskohli, guggulsterone and many others that contain constituents that activate PXR [19].

Tian xian (also known as Tien Hsein and pronounced "Dianne Sean") products are herbal dietary supplements manufactured in China by the China-Japan Feida Union Co., Ltd. (www.cjfu.com/en/Main.php). Tian xian products are distributed world-wide and are aggressively marketed as anti-cancer herbal therapy through several websites including www.tianxian.co.uk, www.cancer-tian-xian.com, www.original-**tianxian**.com, and www.**tianxian**.com. These products are also marketed as herbal therapies that alleviate the unpleasant side effects associated with

western-style anti-cancer treatments (www.tianxian.com/products/products.asp#3). The main supportive information regarding their therapeutic efficacy as anti-cancer agents comes in the form of online testimonials, many of which can be found as web links to the online distributors of these products (for an example see: www.cancer-central.com/).

Currently there are no published studies or clinical trials in the scientific literature establishing the efficacy of these herbal remedies as treatments for cancer, or for their effectiveness as agents that can reduce the side effects of conventional chemotherapy in patients. However, there are three published studies from one laboratory that were performed at the School of Dentistry in the College of Medicine at National Taiwan University in Taipei, Taiwan on the biological effects of tian xian liquid [20-22]. The authors conclude that a liquid formulation of tian xian modulates antigen-stimulated cytokine production by T-cells isolated from patients with recurrent aphthous ulcerations, inhibits cell growth, and induces apoptosis in a wide variety of human cancer cells in cell-based assays.

Multiple tian xian product lines exist on the market including several powder formulations contained in gelatin capsules, a liquid extract, plaster, suppositories, and an ointment (www.cjfu.com/en/2_Products/). A careful examination of the information available on the websites reveals that the main product marketed as a treatment for cancer patients is derived from the 'original' formulation of Tien-Hsien Capsule No.1. The dosing regimen for this powdered capsule product is three to six capsules three times daily with warm water after meals. The herbal ingredients for

tian xian capsule #1, their proportion, and the purported therapeutic effect can be found at the manufacturers' website (http://www.cjfu.com/en/2_Products/). The herbs and their proportions are listed here: Radix Trichosanthis (10%), Radix Clematidis (10%), Radix Ginseng (15%), Radix Astragali seu Hedysari (10%), Ji Xing Zi (10%), Venenum Bufonis (3%), Radix Gentianae (7%), Caculus Bovis (5%), Polyporus Umbellatus (10%), and Radix Pulsatillae (20%). The high potential for herb-drug interactions in patients undergoing conventional chemotherapy is of great concern. We therefore sought to determine the extent to which these agents could potentially alter the pharmacokinetic and pharmacodynamic properties of co-administered CYP substrates.

Here, we use cell-based reporter gene assays and primary cultures of rodent and human hepatocytes to determine the extent to which an extract of tian xian produces alterations in the expression of CYP3A, a clinically important anti-cancer drug metabolizing enzyme in liver. We also describe the creation, validation, and use of a novel line of genetically engineered transgenic mice that express a FLAG-tagged human PXR protein selectively in the liver of mice lacking the murine *Pxr* gene.

3.2 Materials and Methods

Animal Care. All rodents were maintained on standard laboratory chow and allowed food and water ad libitum. The studies reported here have been carried out in accordance with the Declaration of Helsinki and/or with the Guide for the Care and Use of Laboratory Animals as adopted and promulgated by the U.S. National Institutes of Health.

Compounds and Plasmids. Unless otherwise stated, all chemical compounds were purchased from Sigma (St. Louis, MO). The pSG5-hPXR and the pSG5-mPXR was previously described [23]. The GAL4-SRC1, GAL4-PBP, and GAL4-NCoR1 expression vectors were previously described [23]. The full-length human PXR was fused to the VP16 transcriptional activation domain as described [23]. The pFR-LUC reporter gene which is responsive to GAL4-fusion proteins is commercially available (BD Biosciences, Palo Alto, CA). The pFLAG-hPXR vector was constructed by excising the human PXR cDNA from pSG5-hPXR using EcoRI and SalI sites and inserting it into pCMV-TAG2B vector (Stratagene, La Jolla CA).

Extract Preparation. An extract of tian xian Capsule No.1 (Green and Gold International, Manilla, Phillipines) was prepared using one capsule (250 mgs powder) and 1 ml of absolute ethanol. The mixture was placed in a 1.5 ml centrifuge tube and extracted overnight at 4°C on a rotating shaker. The mixture was centrifuged at 16,000 X g for 5 min. The ethyl alcohol supernatant was decanted and kept at -20^{o}C until use.

Cell Culture and Transient Transfection Analysis. The XREM-LUC reporter gene assays were performed as described [24]. The mammalian two-hybrid system analysis was performed as previously described [23].

Generation of a TTR-FLAG-Tagged hPXR Mini-gene. The plasmid containing the TTR mini-gene shown in figure 3-3A was digested with Stu I and subsequently treated with calf intestinal alkaline phosphatase. The FLAG-tagged human PXR cDNA was excised from pFLAG-hPXR using NotI and XhoI, treated

with Klenow DNA polymerase and dNTPs, and was ligated together with the StuI digested pTTR mini-gene. A graphical representation of the TTR-FLAG-tagged human PXR transgene is shown in figure 3-3A.

Transgenic Mouse Production and Genotyping. The TTR-FLAG-tagged hPXR transgene was excised with HindIII. The resulting 6 kb fragment was gel-purified using the QIAEX II (Qiagen, Valencia, CA) DNA purification kit. The transgene was then injected into single-cell B6C3f1 mouse zygotes. Transgene positive mice were screened using polymerase chain reaction. Briefly, a forward primer derived from the TTR promoter (5' cctggtgcacagcagtgcatc 3') and a reverse primer derived from human PXR (5' cctccgacttcctcatctgcg 3') were used to amplify a 424 bp sequence that would not be present in wild-type mice. Cycling conditions used for the genotyping reactions were as follows: 95°C for 15 seconds, 65°C for 15 seconds, and 68°C for 15 seconds for 35 cycles.

Detection of hPXR Protein and Expression-profiling of the hPXR Transgene. Approximately 250 milligrams of liver tissue was homogenized using a dounce Teflon homogenizer in 3 ml of lysis buffer containing 50 mM Tris-HCL pH 7.4, 150 mM NaCl, 1 mM EDTA, and 1% Triton X-100 and protease inhibitors. The homogenate was placed in a centrifuge at 2500 x g for 10 min. The supernatant was pre-cleared using 20 μl protein-A agarose. The resulting supernatant was immunoprecipitated using agarose linked to the M2 monoclonal antibody that recognizes the FLAG epitope. Following SDS-PAGE, the proteins were transferred to nitrocellulose membrane that was probed with our anti-hPXR antibody.

To determine expression profile of the FLAG-tagged human PXR transgene, RNA was isolated from the heart, lung, and liver using wild type and transgenic mice as described [8]. Following DNase I treatment, 1μg of RNA was reverse transcribed and real-time quantitative polymerase chain reaction was performed to detect the human PXR transgene (left primer- 5' caggaggaaattgatgcagtttt 3'; right primer- 5' gtcaagatactccatctgtagcacagt; fluorogenic probe- 5' cccaataaggcaccacccacctatga 3'). All values were normalized to signal from 18S (left primer- 5' ccagtaagtgcgggtcataa 3'; right primer 5' ggttcacctacggaaacctt 3'; fluorogenic probe- 5' cgattggatggtttagtgaggccc 3' as described [10].

'Humanized' PXR mouse production. PXR-knockout (PXR-KO) mice were generated as described previously [9]. The transgenic mice harboring the FLAG-tagged hPXR mini-gene were crossed with the PXR-KO mice to obtain a mouse line expressing human PXR in a PXR-KO mouse background (TTR-hPXR). Following successful generation of 'humanized' mice lacking the mouse *Pxr* gene, the transgenic mice were backcrossed to C57Bl6 mice and then intercrossed to generate a homozygous and congenic line of mice that express the FLAG-tagged hPXR gene selectively in liver.

Statistical Analyisis. Differences between reporter gene and messenger RNA levels were determined using a one-way ANOVA followed by the Duncan's multiple range post-hoc tests.

3.3 Results

Activation of PXR by Tian Xian in Cell-based Assays. To determine the extent to which the extract of tian xian activated human PXR we used a previously described cell-based reporter gene assay [24]. In CV-1 cells, 10 μM rifampicin (RIF) activated the XREM-LUC reporter gene in the presence of transfected human PXR (Figure 3-1). A stock extract of tian xian (250 mg/ml) was used to treat transfected CV-1 cells. A clear concentration-response was also observed with increasing amounts of tian xian extract (Figure 3-1). Thus, in CV-1 cells an extract of tian xian produced efficacious activation of human PXR. The activation of PXR by tian xian at the highest concentrations examined was comparable to that of 10 μM rifampicin, a well known PXR ligand.

Figure 3-1

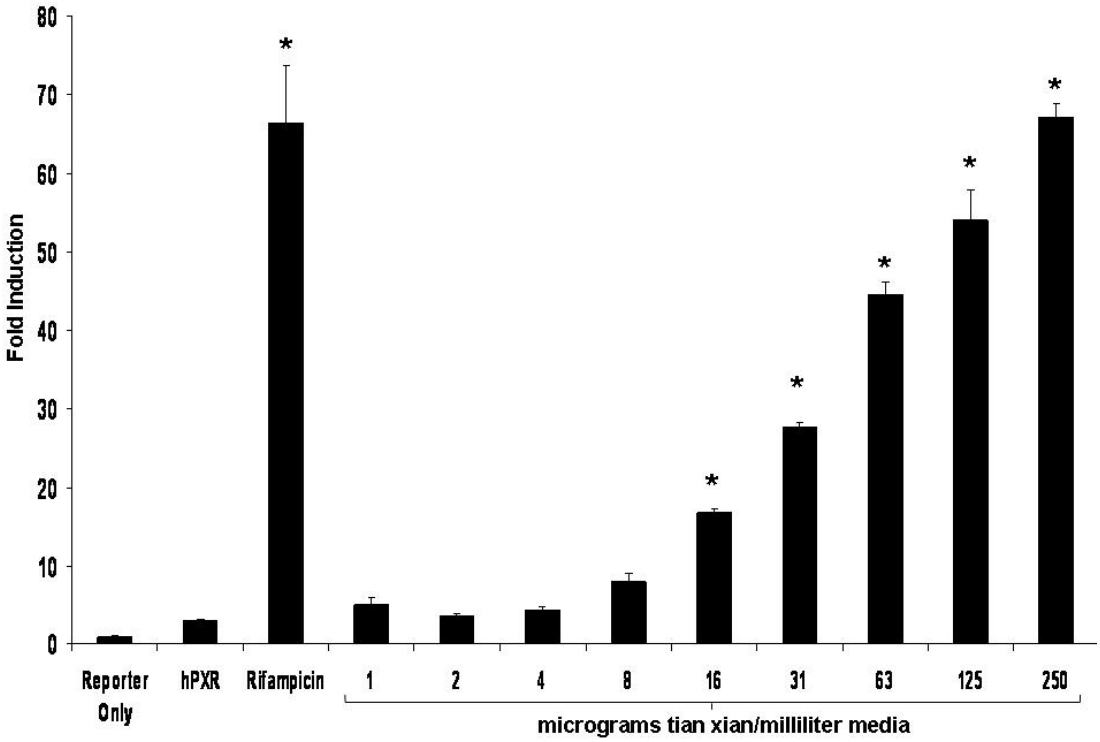

Figure 3-1. Tian Xian Induces PXR Activity in XREM-Luc Reporter Gene Assays. (A) CV-1 cells were transfected with the expression vector for human PXR and the CYP3A4-derived XREM-Luc reporter gene. Cells were treated with vehicle (Veh, 0.1% Ethanol) or 10 μM rifampicin. Two-fold serial dilutions of a stock extract of tian xian (250 mg/ml) were used to perform the concentration-response analysis. All compounds were added as 1000X stock to each individual well. All cells were treated for 24 h. The data represent the mean of replicates $\pm$ SD (n=8) and are normalized against β-galactosidase activity, and are expressed as fold induction over vehicle control. * = Statistically different from vehicle control (p<0.05).

Modulation of PXR-cofactor Interactions in the Mammalian Two-hybrid System. The interaction between accessory protein cofactors and PXR is modulated by the presence of activating ligands in cells. Specifically, in the absence of activating ligands PXR exhibits a strong association with the nuclear receptor co-repressor protein - NCoR [25]. Conversely, in the presence of activating ligands PXR strongly associates with members of the steroid receptor coactivator family including SRC-1 and SRC-2 [26, 27]. CV-1 cells were transfected with expression vectors encoding the GAL4 DNA-binding domain fused to the respective nuclear receptor-interacting domains in the co-activator proteins SRC-1 and SRC-2 together with an expression vector encoding VP16-tagged full-length human PXR. The GAL4-responsive luciferase reporter gene, pFR-LUC, was used to determine the extent to which tian xian extract modulated interaction between human PXR and protein cofactors in cell-based assays. Similar to rifampicin, treatment with increasing concentrations of tian xian extract (4, 31, and 250 μg/ml) recruited VP16-tagged human PXR to GAL4-SRC-1 and GAL4-SRC-2 (Figure 3-2A), but displaced VP16-tagged human PXR from GAL4-NCoR (Figure 3-2B). These data strongly suggest that the extract of tian xian contains biologically active molecules that modulate PXR-co-factor interactions in cell-based assays. While this is useful information, activation of human PXR in a reporter gene assay does not always correlate with the ability to activate PXR in the context of hepatocytes.

Figure 3-2A

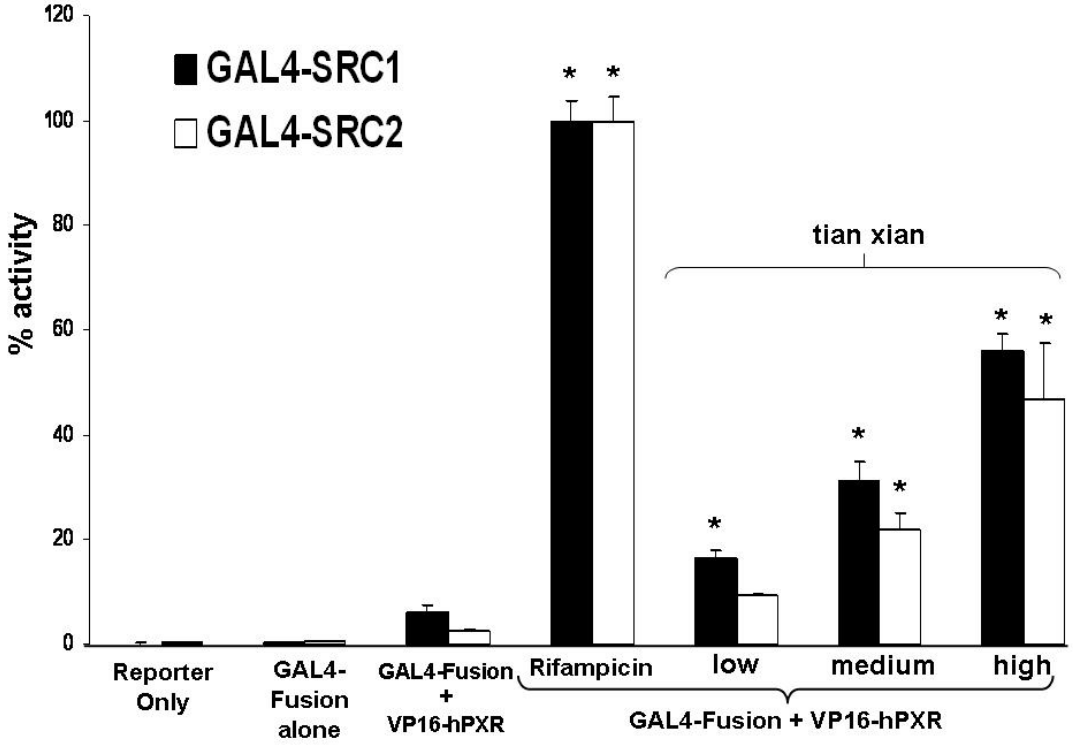
GAL4-SRC1
GAL4-SRC2
% activity
120
100
80
60
40
20
0
tian xian
Reporter Only
GAL4-Fusion alone
GAL4-Fusion + VP16-hPXR
Rifampicin
low
medium
high
GAL4-Fusion + VP16-hPXR

Figure 3-2B

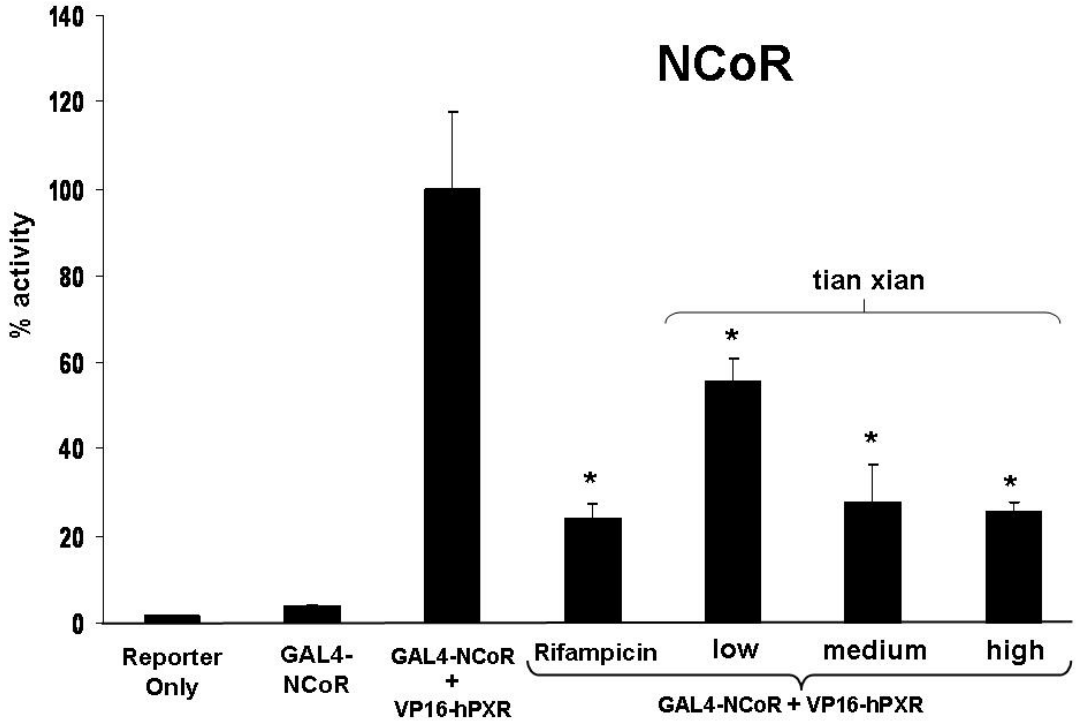

Figure 3-2. Differential Modulation of PXR-SRC-1/2 and PXR-NCoR Interactions by Tian Xian. Receptor-interaction domains fused to the GAL4-DNA-binding domain were used to determine whether tian xian altered PXR association with **(A)** SRC1/2 and **(B)** NCoR in the mammalian two-hybrid system. Transient transfection of CV-1 cells was performed as described in materials and methods. Twenty-four hours post-transfection, CV-1 cells were treated with vehicle (Veh, 0.1% Ethanol) or 10 μM rifampicin. A stock extract (250 mg/ml) was used to treat cells with three different dilutions of tian xian (1:64,000; 1:8,000, and 1:1,000). All compounds were delivered as 1000X (1 μl/ml) and all wells were treated for 24 hours. The data represent the mean of replicates $\pm$ SD (n=8) and are normalized against β-galactosidase activity, and are expressed as percent full reporter gene activity. In (A) * = Statistically different from GAL4 fusion alone control (p<0.05). In (B) * = Statistically different from GAL4-NCoR + VP16-hPXR control (p<0.05).

Construction and In Vivo Hepatic Expression of a FLAG-tagged Human PXR Mini-gene in PXR Knockout Mice. The best characterized PXR-target gene in mouse liver encodes the Cyp3a11 enzyme, the heme-containing steroid mono-oxygenase and functional orthologue of human CYP3A4. We have produced a novel line of transgenic mice in which expression of the FLAG-tagged human PXR cDNA is under the control of an enhancer region isolated from the transthyretin promoter. As shown in figure 3-3A, this transgene drives expression of the FLAG-tagged human PXR transgene in a liver-selective manner. This is consistent with the previous use of the same transthyretin enhancer region in other lines of transgenic mice [28]. We subsequently crossed this line of mice with the previously described PXR knockout mice [9], and then back-crossed these mice into the C57BL/6 line of mice to create a novel strain of mice that are homozygous for the transgene and lack the murine *pxr* gene. Using this strategy we have created a novel line of mice that express the FLAG-tagged human PXR protein in a liver-specific manner in the absence of the murine *Pxr* gene (Figure 3-3B).

Figure 3-3A

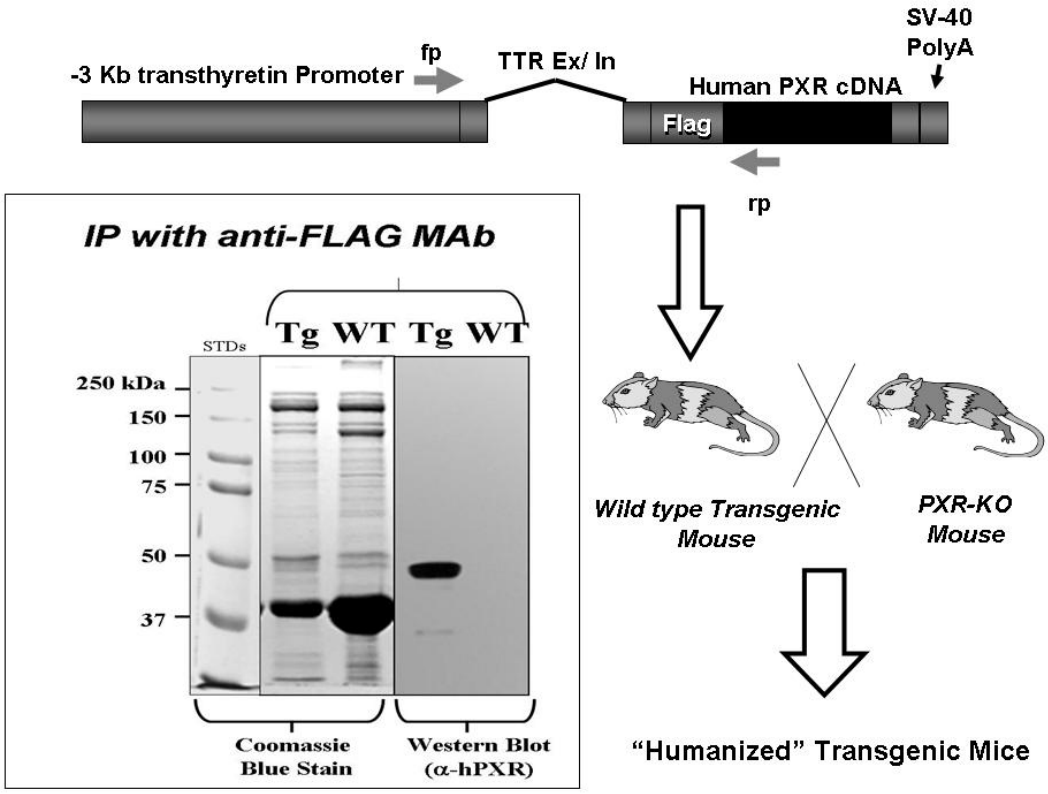
SV-40
PolyA
-3 Kb transthyretin Promoter
fp
TTR Ex/ In
Human PXR cDNA
Flag
rp
IP with anti-FLAG MAb
Tg WT Tg WT
STDs
250 kDa
150
100
75
50
37
Coomassie Blue Stain
Western Blot (α-hPXR)
Wild type Transgenic Mouse
PXR-KO Mouse
"Humanized" Transgenic Mice

Figure 3-3B

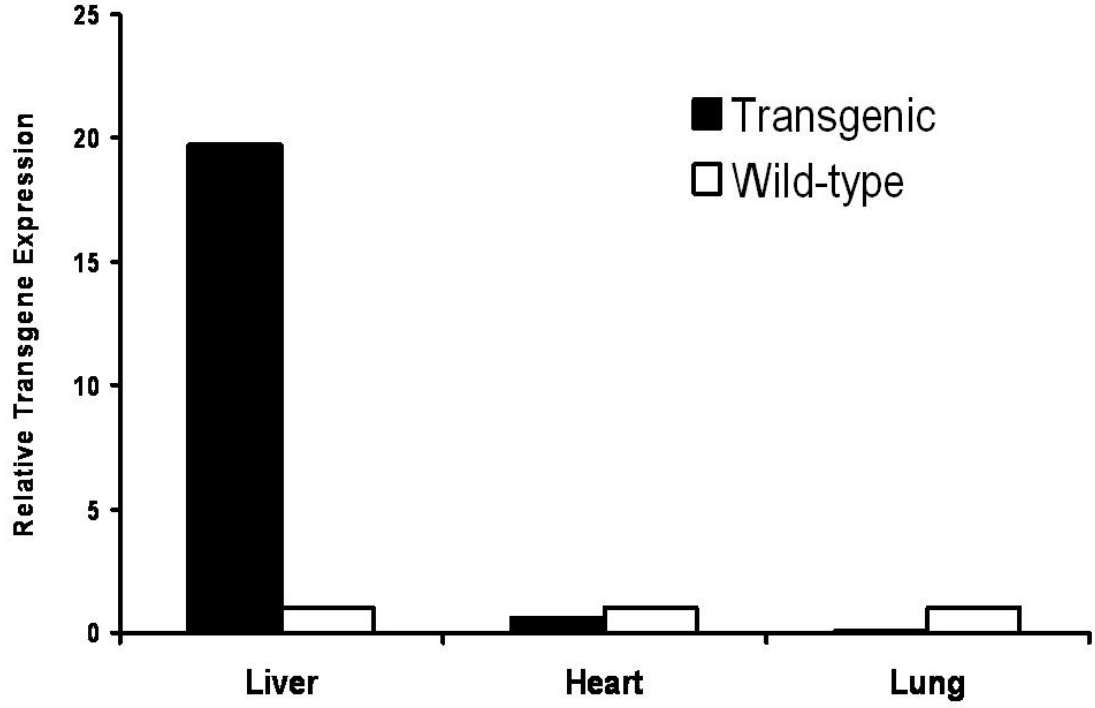

Figure 3-3. Humanized PXR Transgenic Mouse Production and Expression Profiling. (A). A 3,000 bp upstream fragment of the transthyretin promoter was used to drive expression of the FLAG-tagged human PXR cDNA. The location of the PCR primers used for genotyping is shown with the forward primer (fp) located in the transthyretin promoter region. The reverse primer (rp) is derived from the human PXR cDNA sequence. The sequence of each primer is listed in Materials and Methods. The resulting transgenic mouse line was crossed to the PXR knockout mice and then bred to homozygosity for both the *Pxr* knockout allele as well as the transgenic allele. Nine successive backcrosses were performed into the C57Bl6 strain of mice to obtain a congenic line of mice that are *homozygous* for the transgene and *nullizygous* for the wild type *Pxr* allele. The anti-FLAG M2 monoclonal antibody was used to precipitate immuno-reactive proteins from wild type and humanized PXR transgenic livers. Proteins were resolved using SDS-PAGE on identical gels. One was stained with coomassie blue (left) and the other was transferred to PVDF membrane. The membrane was subsequently probed using anti-human PXR antibodies (right). **(B).** Total RNA was isolated from liver, heart, and lung tissue. The RNA was DNase-treated and reverse transcribed as described in Materials and Methods. Real-time quantitative PCR analysis was used to detect expression levels of the human PXR transgene. Data are expressed as relative transgene expression over wild type liver control and are normalized to 18S values obtained as described in Materials and Methods.

The 'Humanized' PXR Transgenic Mice Exhibit Species-specific Responses to Known Species-specific PXR Activators. It is well known that PXR exhibits a species-specific response to certain CYP3A inducers [4]. Indeed, several humanized PXR transgenic mouse models have already been developed that are currently being used to assess the potential for drug-drug interactions commercially and in academic laboratory settings [29, 30]. The hallmark experiment that determines the utility of these mouse models is the administration of rifampicin, a selective human PXR activator, and pregnenolone 16α carbonitrile (PCN), a selective mouse PXR activator to distinguish the functional difference between wild type and humanized PXR mice. We therefore administered 10 μM concentrations of these two compounds for 48 hours to primary cultures of hepatocytes isolated from wild type, PXR-KO, and humanized PXR mice (Figure 3-4A). As expected, PCN induced the expression of *Cyp3a11* in wild type hepatocytes, while rifampicin had only a minimal effect. Also as expected, neither rifampicin nor PCN had any effect on the expression of *Cyp3a11* in hepatocytes isolated from PXR-KO mice. In contrast, treatment of primary cultures of hepatocytes isolated from humanized PXR mice with rifampicin produced marked induction of *Cyp3a11* gene expression, while treatment with PCN produced only minimal increased expression of this known PXR-target gene.

To determine the extent to which PXR activity is required for induction of *Cyp3a11* gene expression we treated primary cultures of hepatocytes isolated from wild type and PXR-KO mice with 10 μM PCN and increasing concentrations of tian xian (4, 31, and 250 μg/ml). Treatment with PCN produced robust and PXR-

dependent induction of *Cyp3a11* gene expression, while treatment with tian xian also increased *Cyp3a11* gene expression in a concentration- and PXR-dependent manner (Figure 3-4B). Primary cultures of hepatocytes isolated from humanized PXR mice were treated with 10 μM rifampicin and increasing concentrations of tian xian (4, 31, and 250 μg/ml). Tian xian treatment produced increased levels of *Cyp3a11* gene expression in a concentration-dependent manner, similar to that obtained with rifampicin, the prototypical human PXR activator (Figure 3-4C). These data indicate that compounds contained within the tian xian extract activate both mouse and human PXR and produce increased expression of a known PXR-target gene, *Cyp3a11*, in the context of cultured hepatocytes.

Figure 3-4A

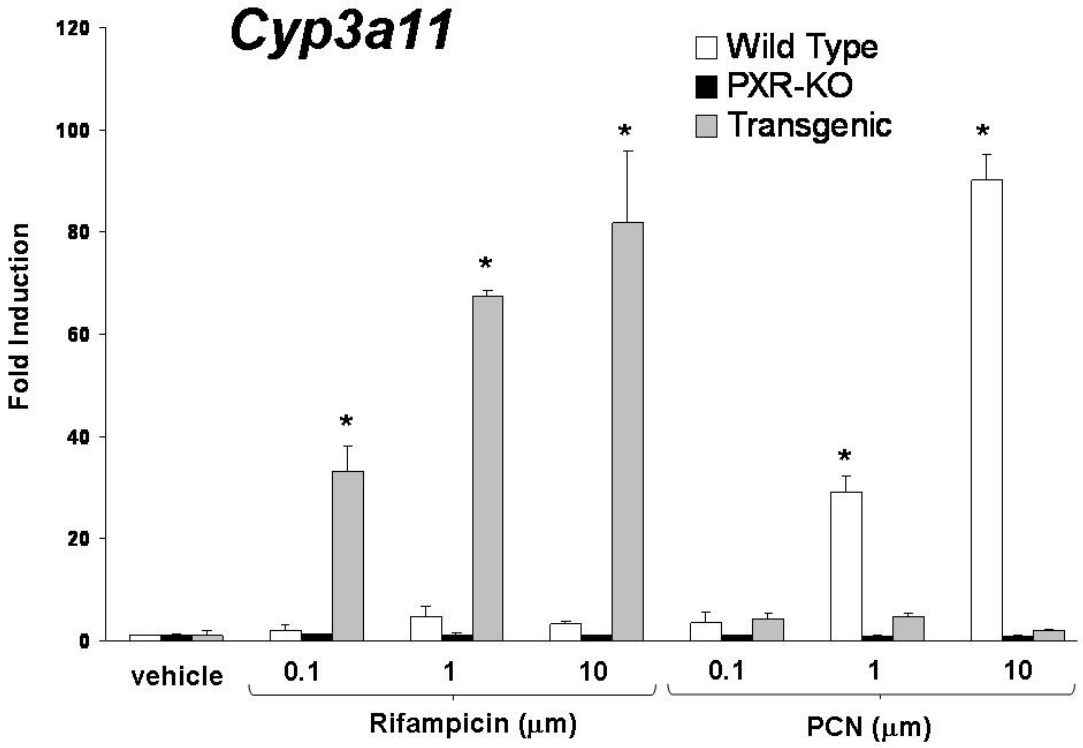
Cyp3a11
Wild Type
PXR-KO
Transgenic
Fold Induction
120
100
80
60
40
20
0
*
vehicle
0.1
1
10
Rifampicin (μm)
0.1
1
10
PCN (μm)

Figure 3-4B

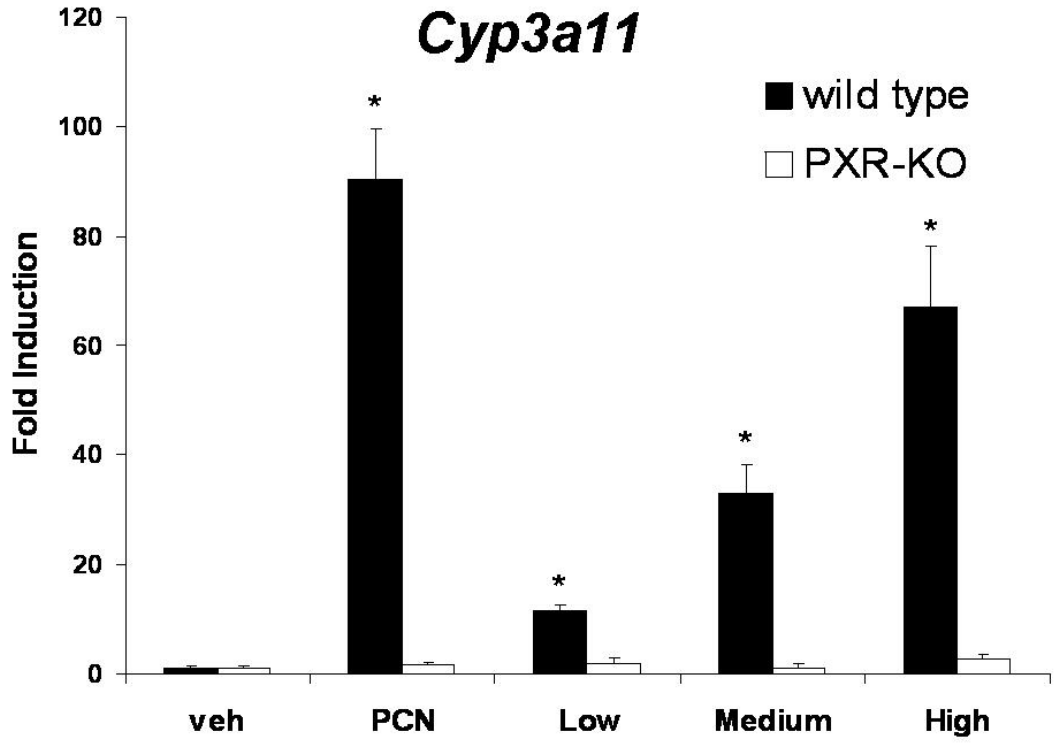
Cyp3a11
wild type
PXR-KO
Fold Induction
120
100
80
60
40
20
0
*
*
*
*
veh
PCN
Low
Medium
High

Figure 3-4C

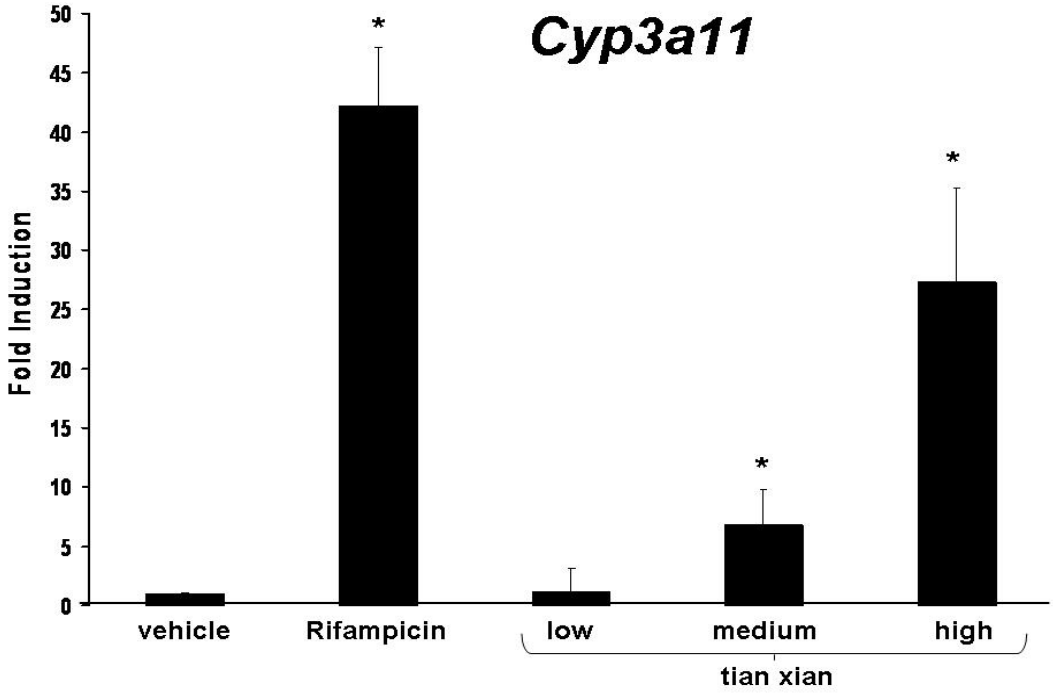

Figure 3-4. The Expression of *Cyp3a11* is Induced by Tian Xian in a PXR-dependent Manner and in Humanized PXR Mouse Hepatocytes. (A). Primary cultures of hepatocytes were isolated from transgenic humanized PXR, PXR knockout, and wild type mice. Cultures were treated with vehicle (Veh, 0.1% DMSO) or 10 μM of Rifampicin or PCN. All cells were treated for 48 hours before RNA isolation. Total RNA was isolated and used in real time quantitative PCR analysis. The data are normalized to 18S levels and are expressed as average values (n=3) ± SD. * = Statistically different from vehicle control group (p<0.05). **(B).** Primary cultures of hepatocytes were isolated from wild type and PXR-KO mice. Cultures were treated with vehicle (Veh, 0.1% DMSO), 10 μM PCN, and increasing concentrations of tian xian extract. All cells were treated for 48 hours before RNA isolation. Total RNA was isolated and used in real time quantitative PCR analysis. The data are normalized to 18S levels and are expressed as average values (n=3) ± SD. * = Statistically different from vehicle control group. **(C).** Primary cultures of hepatocytes were isolated from transgenic humanized PXR mice. Cultures were treated with vehicle (Veh, 0.1% Ethanol), 10 μM of Rifampicin, or with three different dilutions of tian xian (1:64,000; 1:8,000, and 1:1,000). All compounds were delivered as 1000X (1 μl/ml) and all wells were treated for 24 hr. All cells were treated for 24 h before RNA isolation. Total RNA was isolated and used in real time quantitative PCR analysis. The data are normalized to 18S levels and are expressed as average values (n=3) ± the SD. * = Statistically different from vehicle control group.

Tian Xian Induces the Expression of CYP3A4 in Primary Cultures of Human Hepatocytes. The relative expense and low availability of primary cultures of human hepatocytes has recently led to a large effort to find suitable alternatives to test the potential for drug-drug and herb-drug interactions. One of the more positive aspects of the PXR reporter gene assay and the use of humanized mouse models includes the genetic uniformity and technical convenience of both cell-based systems and engineered mouse models. However, the 'gold standard' of drug metabolism studies required by the Food and Drug Administration (FDA) in the United States still remains the use of primary cultures of human hepatocytes. We therefore sought to determine the extent to which the expression of the *CYP3A4* gene was altered by administration of tian xian. Figure 3-5 reveals that treatment of primary cultures of human hepatocytes with increasing concentrations of tian xian produced a concentration-dependent increase in the expression of the important drug metabolizing enzyme CYP3A4.

Figure 3-5

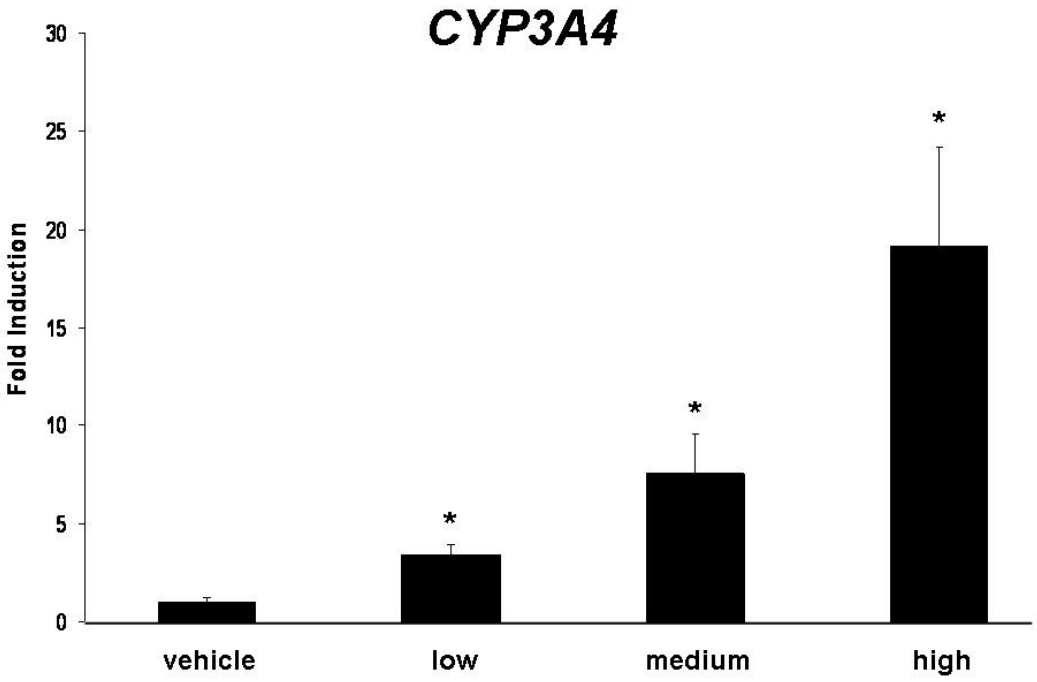

Figure 3-5. The Expression of CYP3A4 is Induced Tian Xian in Hepatocytes Isolated from the Transgenic Humanized PXR Mice. Primary cultures of hepatocytes were obtained from XenoTech, LLC. Cultures were treated with vehicle (Veh, 0.1% Ethanol), or with three different dilutions of tian xian (1:64,000; 1:8,000, and 1:1,000). All compounds were delivered as 1000X (1 μl/ml) and all wells were treated for 24 hr. All cells were treated for 24 h before RNA isolation. Total RNA was isolated and used in real time quantitative PCR analysis. The data are normalized to 18S levels and are expressed as average values (n=3) $\pm$ SD. * = Statistically different from vehicle control group.

3.4 Discussion

It has been nearly twenty years since the identification of drug-inducible members of the CYP3A subfamily of drug metabolizing enzymes [31-33]. It is now well known that induction of *CYP3A4* gene expression in liver and intestine at the level of transcription by nuclear receptor proteins produces clinically relevant elevations in enzymatic activity of this extremely important drug-metabolizing enzyme [12]. It is also well known that both drug-drug and herb-drug interactions can affect the clinical outcome in cancer patients on combination therapy [34]. The purpose of this study was to determine the extent to which treatment with tian xian has the potential to produce alterations in the expression and activity of CYP3A4 in human patients. Moreover, we present here a novel humanized PXR mouse model that will undoubtedly be useful for future studies involved in the pre-clinical testing of candidate drug molecules and additional herbal remedies.

There are two previously described transgenic 'humanized' PXR mouse models. The first transgenic mouse model created utilized the albumin promoter to drive expression of the human PXR cDNA selectively in liver, but this model subsequently lacked any expression of the transgene in intestine [30]. To compensate for this lack of intestinal expression, another transgene was engineered using the fatty acid-binding protein promoter, thus generating a bi-transgenic mouse model with expression in both liver and intestine [35]. Recently, another group took a different approach that utilized a bacterial artificial chromosome containing the entire human *PXR* gene, including the relevant promoter regulatory sequences, to drive expression

of potentially all PXR splice variants in a manner that more closely recapitulates *PXR* expression in humans [36]. The model we present here has utilized the transthyretin promoter that expresses a FLAG-tagged human PXR cDNA selectively in liver and choroid plexus in brain (data not shown) in our PXR-KO mouse model, and has been backcrossed to produce a congenic line of mice containing the C57Bl6 genetic background.

Humanized mouse models are becoming extremely important in the pre-clinical testing of novel drug candidate molecules. By knocking-out the rodent *pxr* gene and replacing it with the human receptor, 'humanized' PXR mouse models have been established as unique tools to dissect the drug-induced xenobiotic response, and are aiding the development of safer drugs at an earlier stage of pre-clinical drug development. These unique mouse models all have the advantage of providing reliable, cost-effective, plentiful, convenient, and genetically uniform systems that can be used to test for potential drug-drug and herb-drug interactions. This is of particular importance in the herbal remedy industry, as it is not currently required by the FDA in this country to determine the extent to which their products are safe for co-administration with concurrently used prescription medications.

Tian xian represents only one example of potential herb-drug interactions, but we feel that the experiments presented here are particularly important because this herbal remedy is marketed on the world-wide web and is available without a prescription as an anti-cancer therapy to be used in conjunction with ‘western’ chemotherapeutic agents. This product line purports to validate its efficacy as an

anti-cancer herbal remedy using on-line 'testimonials'. These testimonials tend to be cancer patients that are not responding to their conventional chemotherapy, but testify that tian xian co-administration eased their side-effects and increased the effectiveness of their chemotherapy. Cancer patients can not test the validity of these on-line claims before using tian xian, and the experiments presented here do not specifically refute these claims. However, our data strongly suggest that co-administration of tian xian together with conventional chemotherapeutic agents, many of which are indeed substrates of the CYP3A4 enzyme, would likely increase their biotransformation. The potential danger highlighted in this study is that co-administration of tian xian and conventional chemotherapeutics would tend to decrease the efficacy of anti-cancer agents that are metabolized and excreted by PXR-dependent mechanisms. Conversely, co-administration of tian xian with a pro-drug that requires bioactivation would likely increase the rate of such a conversion. As is the case with cyclophosphamide and ifosfamide, induction of CYP3A4 activity would likely promote accumulation and possible toxicity due to their narrow therapeutic index. Finally, since the tian xian extract is a complex mixture of compounds, it is a formal possibility that tian xian could simultaneously activate PXR and inhibit CYP enzymatic activity. Future studies should address this issue by examining alterations in cytochrome P450 activity following administration of this herbal remedy.

It is well known that activation of PXR coordinately regulates the expression and activity of multiple drug transporter proteins, as well as numerous other drug metabolizing enzymes. In addition to CYP3A4, PXR is involved in regulating

numerous members of the UDP-glucuronosyltransferase family [13, 37], sulfotransferases [15], drug transporter proteins and many other enzymes involved in handling oxidative stress in cells [6, 10, 16, 17]. Future studies will involve determining the extent to which this Chinese herbal remedy modulates the expression and activity of these enzymes in liver using primary cultures of human hepatocytes and the line of transgenic mice described here.

3.5 References

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Chapter 4: Cyclic Amp-Dependent Protein Kinase Signaling Modulates Pregnane x Receptor Activity in a Species-Specific Manner

4.1 Introduction

The nuclear hormone receptor, pregnane x receptor (PXR, NR1I2), regulates drug-inducible gene expression in liver and intestine [1]. PXR is activated by a vast array of compounds including certain steroids and bile acids, a plethora of naturally occurring compounds, specific antibiotics, antifungal drugs, polychlorinated binphenyls, organochloride pesticides, and phenobarbital (PB) [2]. The prototypical marker of PXR activation and best-characterized PXR-target gene in mammals encodes certain members of the CYP3A family of cytochrome P450 (CYP) drug metabolizing enzymes [3, 4]. It is now clear that PXR-mediated gene activation coordinately regulates a group of genes that encode CYP proteins and additional drug metabolizing enzymes, as well as drug transporter proteins in liver and intestine [5]. Hence, PXR-mediated gene activation produces profound up-regulation of the metabolism, transport, and elimination of potentially toxic chemicals including many steroids, xenobiotics, cholesterol metabolites and other compounds from the body.

Ligand-mediated activation of PXR occurs in a species-specific manner [6]. One of the most effective activators of human PXR is the macrocyclic antibiotic, rifampicin. Interestingly, rifampicin does not appreciably activate mouse PXR. Conversely, pregnenolone 16α carbonitrile (PCN) is an efficacious activator of mouse PXR, but has only minimal effect on human PXR. The species-specific

induction of *CYP3A* gene expression can be fully accounted for by evolution of the ligand-binding pocket of this nuclear receptor from mice to humans. We and others have demonstrated this experimentally using novel lines of PXR-knockout mice crossed with additional novel lines of transgenic mice expressing the human PXR protein [7-10]. While much is known regarding the identity of target genes and ligands for this nuclear receptor, very little is known regarding the signal transduction pathways that interface with the PXR protein.

The primary target of intracellular cyclic AMP (cAMP) is cAMP-dependent protein kinase (PKA) [11]. Numerous physiological stimuli such as β-adrenergic stimulation during fasting and caloric restriction, as well as acute inflammation produce increases in the intracellular concentration of cAMP in hepatocytes. The PKA signal transduction pathway is also involved in the phosphorylation of target proteins through indirect interaction with the classical mitogen-activated protein kinase (MAPK) signaling pathway [12]. There are conflicting reports in the literature regarding the effect of PKA signaling on drug-inducible *CYP3A* and *CYP2B* gene expression in hepatocytes. Using primary cultures of rat hepatocytes, Sidhu *et al*. clearly demonstrated that PB-mediated induction of *Cyp3A1* and *Cyp2B1/2* expression is inhibited by treatment with cAMP analogues [13]. Conversely, the same laboratory reported that forskolin treatment produces increased expression of *Cyp3A1* in rat hepatocytes; however induction was independent of cAMP and PKA signaling [14]. The molecular basis for this difference was conclusively demonstrated when two groups independently found that forskolin functions as a

direct agonist of both rodent and human PXR, thereby inducing ligand-dependent expression of *CYP3A* genes [15, 16]. In mice, it has been reported that PB-mediated induction of *Cyp3a11* and *Cyp2b10* gene expression is inhibited by PKA activators, while inhibitors of PKA enhanced drug-inducible *CYP* gene expression [17, 18].

Studies from our laboratory using mouse models show that the PKA signal transduction pathway synergizes with ligand-dependent PXR-mediated induction of *Cyp3a11* gene expression [15]. These studies also revealed that the PKA-mediated synergism is a PXR-dependent phenomenon in mice. Additional studies from our laboratory using mouse models indicate that PKA signaling interfaces with CAR activity by modulating CAR-protein cofactor interactions, and also by increasing the expression of the CAR gene itself [19]. During the course of these studies we noticed significant differences in PKA-dependent alterations of drug-inducible *CYP* gene expression that were dependent upon the species of rodent (mouse -versus- rat) used to isolate primary cultures of hepatocytes. To date, there are no studies that we are aware of that have systematically investigated the effect of PKA signaling upon drug-inducible CYP gene expression in hepatocytes across multiple species. These observations led us to initiate a systematic study of the effect of PKA signaling on drug-inducible *CYP3A* gene expression in cultured hepatocytes across three different species; mice, rats, and humans.

Using primary cultures of hepatocytes we show here that pharmacological activation of PKA signaling using the cyclic AMP analog, 8-bromo cyclic AMP (8-Br-cAMP), has a species-specific effect upon PXR-mediated activation of drug-

inducible *CYP3A* gene expression. In primary cultures of mouse hepatocytes, PKA signaling has a synergistic effect upon PXR-mediated *Cyp3a11* gene activation. Conversely, in primary cultures of human and rat hepatocytes, PKA signaling dramatically represses PXR-mediated drug-inducible *Cyp3A1* gene activation. We use biochemical and pharmacological methods to explore the molecular basis of the interface between the PKA signal transduction pathway and the human PXR protein. *In vitro* ^{32}P-labeling experiments using recombinant human PXR protein and several catalytically active protein kinases reveal that human PXR protein serves as a comparatively effective and direct substrate for PKA. *In vivo* metabolic labeling experiments using ^{32}P-orthophosphate show for the first time that human PXR exists as a phosphoprotein in cells. Moreover, western blot analysis using antibodies directed against phosphothreonine amino acid residues reveals that activation of PKA signaling increases the phosphothreonine content of the human PXR protein. Mammalian two-hybrid analysis indicates that PKA signaling likely represses human PXR activity through increases in the strength of interaction between human PXR and the protein cofactor nuclear receptor co-repressor protein (NCoR). Elucidation of the mechanism by which PKA signaling modulates PXR activity will likely be useful in the prediction and prevention of harmful drug interactions in patients on combination therapy that also suffer from diabetes, obesity, or acute inflammation. It is of further interest to determine the extent to which the interface between PXR and key signal transduction pathways, such as the PKA signaling pathway, is evolutionarily conserved. This thrust of research will also likely be useful in helping to understand

the molecular basis of altered drug metabolism pathways in patients with diabetes, obesity, and metabolic syndrome.

4.2 Materials and Methods

Hepatocyte Culture and Treatment. Hepatocytes were isolated from male mice or rats using a standard collagenase perfusion method as described previously [20]. Hepatocytes were plated in collagen-coated 6-well plates at a density of 8 X 10^5 live cells per well. Primary cultures of human hepatocytes were purchased from CellzDirect (Pittsboro, NC). Forty-eight hours after plating, hepatocytes were treated with 1 millimolar (mM) concentrations 8-bromo-cyclic-AMP or 8-bromo-cyclic-GMP, 10 μM PCN, a known rodent PXR agonist, or 10 μM rifampicin- a known human PXR agonist. Mouse hepatocytes were treated for 24 hours and human hepatocytes were treated 48 hours.

RNA Isolation, Northern Blot and Real-Time Quantitative-PCR Analysis. Total RNA was isolated from cell culture using a commercially available reagent, Trizol (Invitrogen), according to the manufacturer's directions. For Northern Blot analysis, 10 μg total RNA was resolved on a formaldehyde agarose gel. Blots were hybridized with ^{32}P-labeled cDNA corresponding to the cDNA sequences for mouse and rat CYP3A analogs or for 18S RNA as described previously [15, 21]. For QPCR analysis, isolated RNA was DNase treated (Sigma-Aldrich), reverse transcribed (Promega), and quantitative PCR was performed using a Cepheid Smart Cycler™ (Sunnyvale, CA) to detect mRNA expression specific for CYP3A, and CYP2B orthologues in mouse, rat, and human. Primers for CYP3A were designed as follows:

mouse Cyp3a11 (5'-CAA ggA gAT gTT CCC TgT CA and 5'-CCA CgT TCA CTC CAA ATg AT), rat Cyp3A1 (5'-AAT AAg gCA CCT CCC ACC TA and 5'-ggA TCA Cgg TgA AgA gCA TA), and human CYP3A4 (5'-CAg gAg gAA ATT gAT gCA gTT TT and 5'-gTC AAg ATA CTC CAT CTg TAg CAC AGT). Primers for CYP2B were designed as follows : mouse Cyp2b10 (5'-gAC TTT ggg ATg ggA AAg Ag and 5'-CCA AAC ACA ATg gAg CAg AT), human CYP2B6 (5'-AAg Cgg ATT TgT CTT ggT gAA and 5'-Tgg Agg ATg gTg gTg AAg AAg), and rat Cyp2B1/B2 (5'-ggT ACC TgC TTC CCA AgA AC and 5'-ACA AAT gTg CTT TCC TgT gg). Fold induction was calculated using 18S RNA to normalize the data as described previously [22].

Generation of the Human PXR Recombinant Adenovirus. Recombinant adenoviruses were generated using the AdEasy™ Adenoviral Vector System (Stratagene). A *BamH*I/*Xho*I human PXR PCR product was inserted into the pShuttle-IRES-hrGFP1 transfer vector. The PCR primer sequences were 5'-gAC ggC CTC gAg gCT ACC TgT gAT gCC gAA CAA CTC and 5'-gAA ggC CTC gAg gCC ACC ATg gAT TAC AAg gAT gAC. These primers were designed to omit the stop codon in order to fuse the protein to a 3X-FLAG epitope at the COOH-terminus. Viruses were propagated in the AD-293 cell line and were purified using cesium chloride density gradient centrifugation. Cells were transduced with the purified adenovirus expressing FLAG-tagged human PXR at MOI=10.

Immuno-purification of FLAG-tagged Human PXR Protein. Following overnight adenoviral transduction, cells were drug-treated for 24-48 hr and lysed by

sonication in a buffer composed of 50mM Tris-HCL, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton-x, and 1X protease and phosphatase inhibitor cocktails (Thermo Scientific). Cell lysates were precleared with 20 µl of immobilized protein A (Repligen). Immunoprecipitation of the human PXR protein was accomplished using anti-FLAG M2 affinity gel (Sigma-Aldrich) or a polyclonal antibody directed against the human PXR protein as indicated. Control reactions contained non-immune IgG (Sigma-Aldrich) or blank virus as indicated. Free immune complexes were captured with immobilized protein A and and washed three times with lysis buffer.

In Vitro Phosphorylation Analysis. Immuno-purified human PXR protein was subjected to in vitro phosphorylation analysis using catalytically active purified kinases including CDK1, CK2, GSK3 (New England Biosciences), PKA, PKC (Promega), p70S6K, AMPK, and Akt2 (Upstate). Approximately 3 micrograms of human PXR protein was incubated at 30 °C for 30 min with the above kinases and corresponding reaction buffers. Reaction buffer composition was as follows: CDK1 (50mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 2mM DTT, 1mM EGTA, 200µM ATP, 0.5µCi γ ^{32}P ATP), CK2 (20mM Tris-HCl pH 7.4, 80mM KCl, 10mM $MgCl_2$, 200µM ATP, 0.5µCi γ ^{32}P ATP), GSK3 (20mM Tris-HCl pH 7.4, 10mM $MgCl_2$, 5mM DTT, 200µM ATP, 0.5µCi γ ^{32}P ATP), PKA (40mM Tris-HCl pH 7.4, 5mM $MgCl_2$, 200µM ATP, 0.5µCi γ ^{32}P ATP), PKC (20mM HEPES pH 7.4, 10mM $MgCl_2$, 3.4mM $CaCl_2$, 200µM ATP, 0.5µCi γ ^{32}P ATP), p70S6K (40mM MOPS-NaOH pH 7.0, 1mM EDTA, 10mM $MgCl_2$, 0.1mg/ml BSA, 0.01% β-ME, 200µM ATP, 0.5µCi γ ^{32}P ATP), AMPK (30mM HEPES pH 7.4, 10mM $MgCl_2$, 0.2mM DTT, 0.2% NP-40,

300μM AMP, 200μM ATP, 0.5μCi γ ^{32}P ATP), and Akt2 (50mM Tris-HCl pH 7.5, 10mM $MgCl_2$, 0.1mM EGTA, 0.2mM DTT, 200μM ATP, 0.5μCi γ ^{32}P ATP). The samples were subjected to SDS-PAGE. The gel was dried and autoradiography analysis was performed overnight at -80°C.

In Vivo Metabolic Labeling Analysis. HepG2 cells were plated in 6-well plates at a density of 1 x 10^6 cells per well. The cells were transduced with Ad-hPXR or Ad-GFP (MOI=10) overnight. After viral transduction, the cells were treated with phosphate-free DMEM containing 1% dialyzed fetal bovine serum containing 1% Penicillin/Streptomycin for six hours. The culture medium was then supplemented with 300μCi of ^{32}P-orthophosphate per well and treated with vehicle, 1mM 8Br-cAMP, or 1mM 8Br-cGMP for an additional 14 hr. The cells were washed three times in 1X phosphate-buffered saline and human PXR protein was immuno-purified using anti-human PXR antibody as described above. The samples were subjected to SDS-PAGE. The gel was dried and autoradiography analysis was performed for 30 min at room temperature. For control purposes, a duplicate experiment was performed for the parallel western blot analysis that omitted the radiolabel in order to examine the efficiency of immuno-purification.

Detection of Phosphothreonine in Human PXR. The immuno-purified human PXR protein was resolved using 10% SDS-PAGE and transferred to PVDF membranes (Millipore). Western Blot analysis was performed using monoclonal antibodies obtained from the PhosphoDetect™ phosphoserine and phosphothreonine detection kits (Calbiochem). The lambda protein phosphatase (New England

Biolabs) reaction conditions were 50 mM Tris-HCl, 100 mM NaCl, 2 mM Dithiothreitol, 0.1 mM EGTA, 0.01 % Brij 35, 2 mM $MnCl_2$, at pH 7.5 at 25°C.

Transient Transfection and Reporter Gene Analysis. The XREM-Luc and mammalian-2-hybrid reporter gene assays were performed as previously described [20, 21]. Briefly, cells were plated in 96-well plates at a density of 7000 cells per well. After 24 hours the cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The PXR transactivation assays were transfected with 110 ng of DNA per well containing SV40-βgal (40ng), XREM-Luc (20ng), pGFP-hPXR (5ng), pFC-PKA or pFC-MEK1 (10ng), and pBluescript (35ng). The mammalian-2-hybrid assays were transfected with 110 ng of DNA per well containing SV40-βgal (40ng), pFR-Luc (20ng), Gal4-NCoR (20ng), VP16-hPXR (10ng), pFC-PKA (10ng), and pBluescript (10ng). The next day the cells were drug-treated for 24 hours. Luciferase activities were determined using a standard luciferase assay system (Promega). β-galactosidase activities were determined by ONPG assay and were read at 420nm.

Statistical Analyisis. Statistical differences between treatment groups were determined using a one-way ANOVA followed by the Duncan's multiple range post-hoc tests.

4.3 Results

PKA Signaling has a Species-specific Effect on PXR-mediated Gene Activation in Hepatocytes. To determine if PKA signaling has a species-specific effect upon PXR-mediated gene activation, primary cultures of mouse and rat hepatocytes were isolated and treated with vehicle or 10 μM PCN for 24 hr in the presence of increasing concentrations 0.01, 0.1, and 1.0 mM of 8-Br-cAMP and northern blotting analysis was performed (Figure 4-1). As expected, treatment with PCN produced increased expression of both *Cyp3a11* and *Cyp3A1* in primary cultures of mouse and rat hepatocytes, respectively. Strikingly, increasing concentrations of the PKA activator, 8-Br-cAMP, had opposite effects upon PCN-inducible *CYP3A* gene expression in primary cultures of mouse hepatocytes when compared with that obtained using rat hepatocytes. In mouse hepatocytes, 8-Br-cAMP synergized with PCN producing extremely robust levels of *Cyp3a11* gene expression at the highest doses, while in rat hepatocytes it dramatically repressed the expression of *Cyp3A1*. Both the synergistic and repressive effects were concentration-dependent in both species examined, respectively. The synergistic effect of 8-Br-cAMP in mouse hepatocytes was also PXR-dependent, as there was only minimal induction of *Cyp3a11* gene expression at the highest doses of 8-Br-cAMP examined in PXR-knockout mice (data not shown).

Figure 4-1

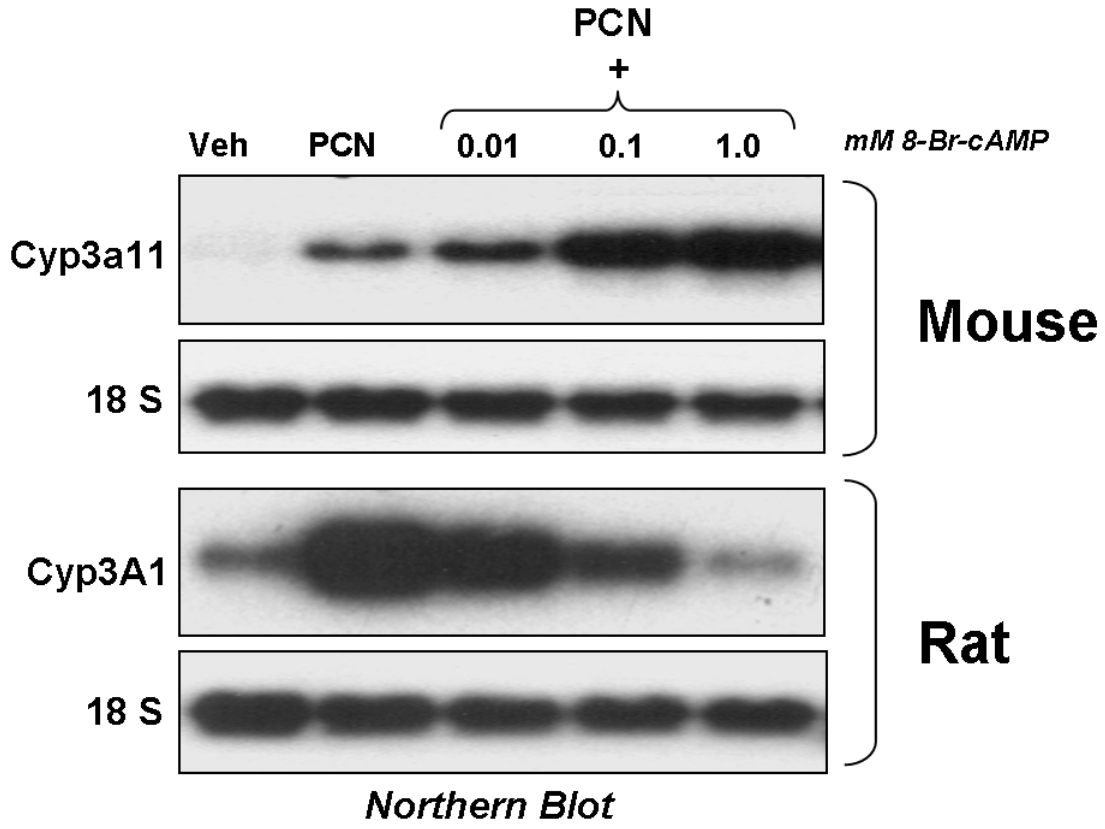


Figure 4-1. PKA activation modulates *CYP3A* gene expression in primary cultures of mouse and rat hepatocytes. Primary rodent hepatocytes were treated with 10 μM PCN and increasing concentrations of 1mM 8Br-cAMP for 24 hours before RNA isolation. Blots were probed sequentially with ^{32}P labeled fragments of CYP3A and 18S.

To further examine the specificity of the effect of the effect of PKA signaling across species we treated primary cultures of mouse, rat, and human hepatocytes for 24 hr with either 8-Br-cAMP, or 8-bromo-cyclic GMP (8-Br-cGMP) as a control compound, in the presence and absence of 10 μM PCN or rifampicin and performed real-time quantitative polymerase chain reaction (Q-PCR) to analyze the expression levels of *CYP3A*. Figure 4-2A shows that treatment of primary cultures of mouse hepatocytes with 1 mM 8-Br-cAMP or 10 μM PCN for 24 hr induced expression of *Cyp3a11* to approximately equivalent levels, while co-treatment with 8-Br-cAMP and PCN produced a synergistic level of PXR-target gene expression (>2000 fold-induction). Treatment with 1 mM 8-Br-cGMP alone had no significant effect upon *Cyp3a11* gene expression levels, and co-treatment with 8-Br-cGMP and PCN produced very similar effects when compared with PCN treatment alone. Primary cultures of rat and human hepatocytes were also treated using identical experimental conditions. Treatment of cultured hepatocytes with 1 mM 8-Br-cAMP isolated from these two species produced the exact opposite effect as that observed with mouse hepatocytes. Figure 4-2B shows that treatment of primary cultures of rat hepatocytes with either 8-Br-cAMP or 8-Br-cGMP produced little or no effect upon *Cyp3A1* gene expression levels, while treatment with 10 μM PCN induced expression as expected. However, treatment of rat hepatocytes with 8-Br-cAMP together with PCN reduced the fold-induction of *Cyp3A1* expression to less than 10% of that observed when compared with PCN treatment alone. In contrast, treatment of rat hepatocytes with 8-Br-cGMP together with PCN induced expression of *Cyp3A1* comparable to that

observed with PCN treatment alone. When primary cultures of human hepatocytes were used, 10 μM rifampicin was substituted for PCN treatment, other than that the experimental conditions were identical. Figure 4-2C reveals that treatment with 8-Br-cAMP produced dramatic repression of rifampicin-inducible *CYP3A4* gene expression. Treatment with 8-Br-cGMP had very little or no effect upon either the basal or rifampicin-inducible expression of the *CYP3A4* gene.

Figure 4-2A

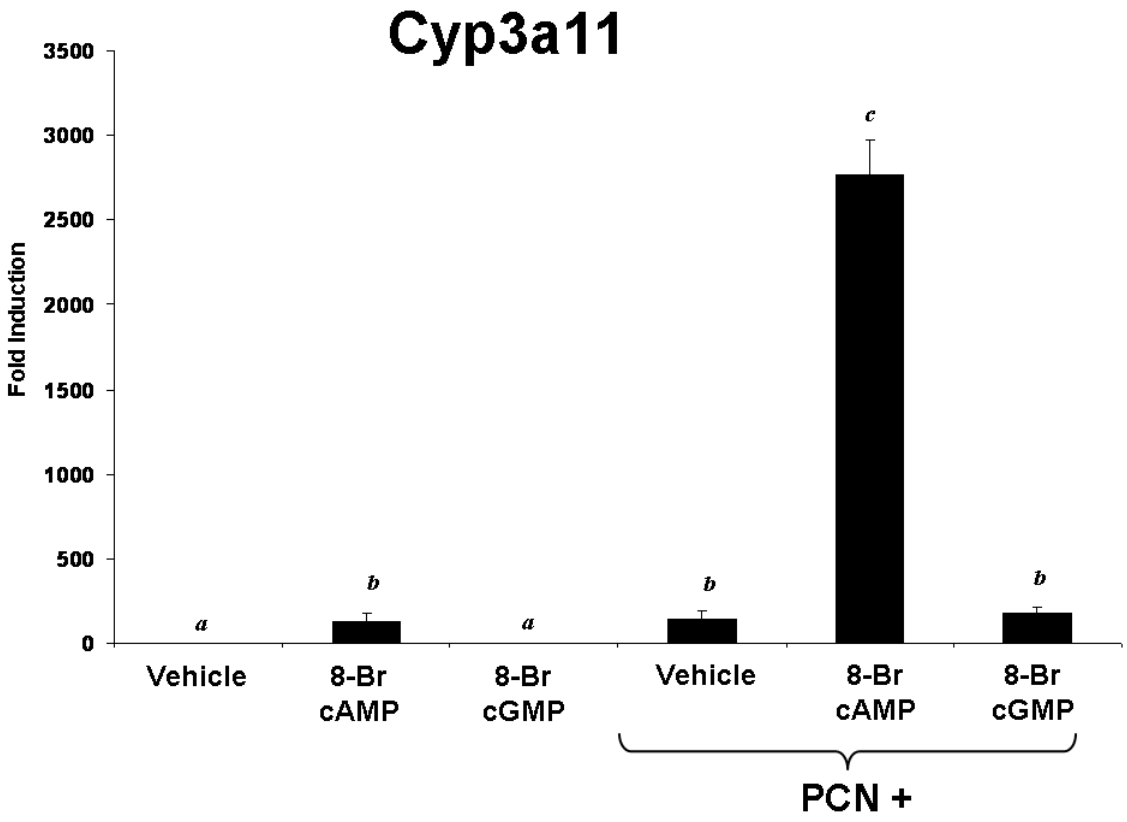

Cyp3a11
Fold Induction
3500
3000
2500
2000
1500
1000
500
0
a
b
a
b
c
b
Vehicle
8-Br cAMP
8-Br cGMP
Vehicle
8-Br cAMP
8-Br cGMP
PCN +

Figure 4-2B

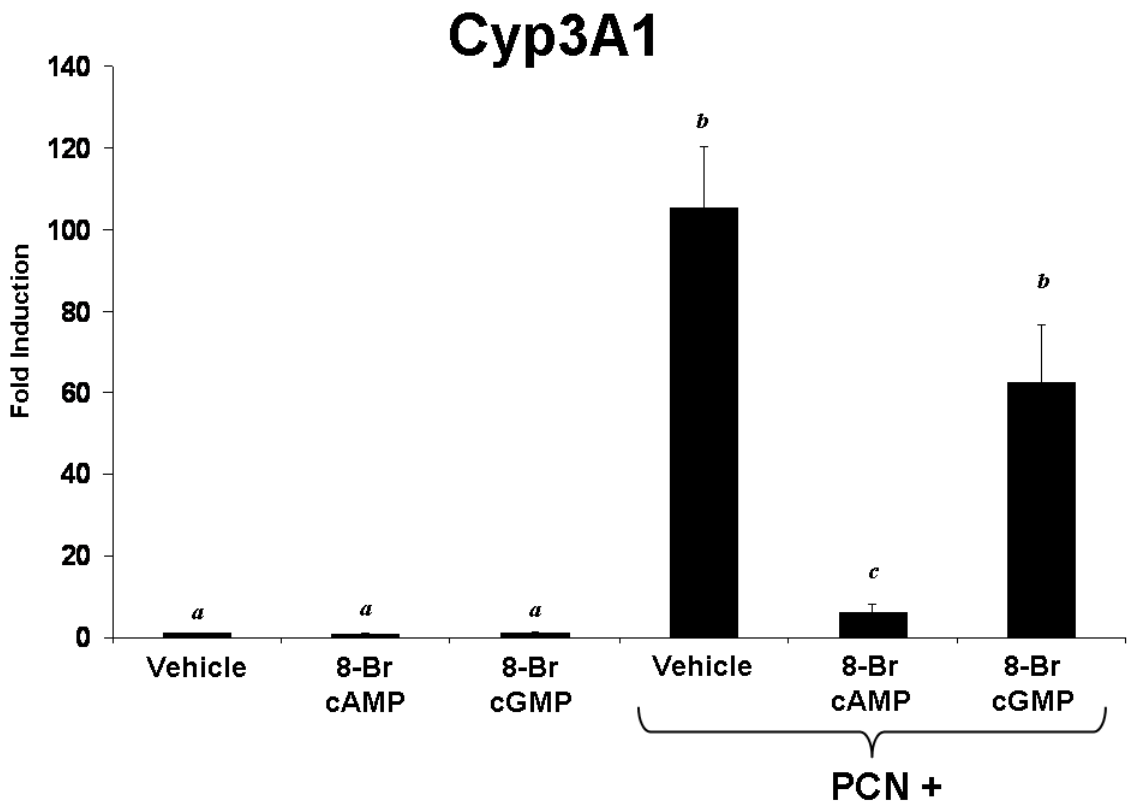

Cyp3A1
Fold Induction
140
120
100
80
60
40
20
0
a
a
a
b
c
b
Vehicle
8-Br cAMP
8-Br cGMP
Vehicle
8-Br cAMP
8-Br cGMP
PCN +

Figure 4-2C

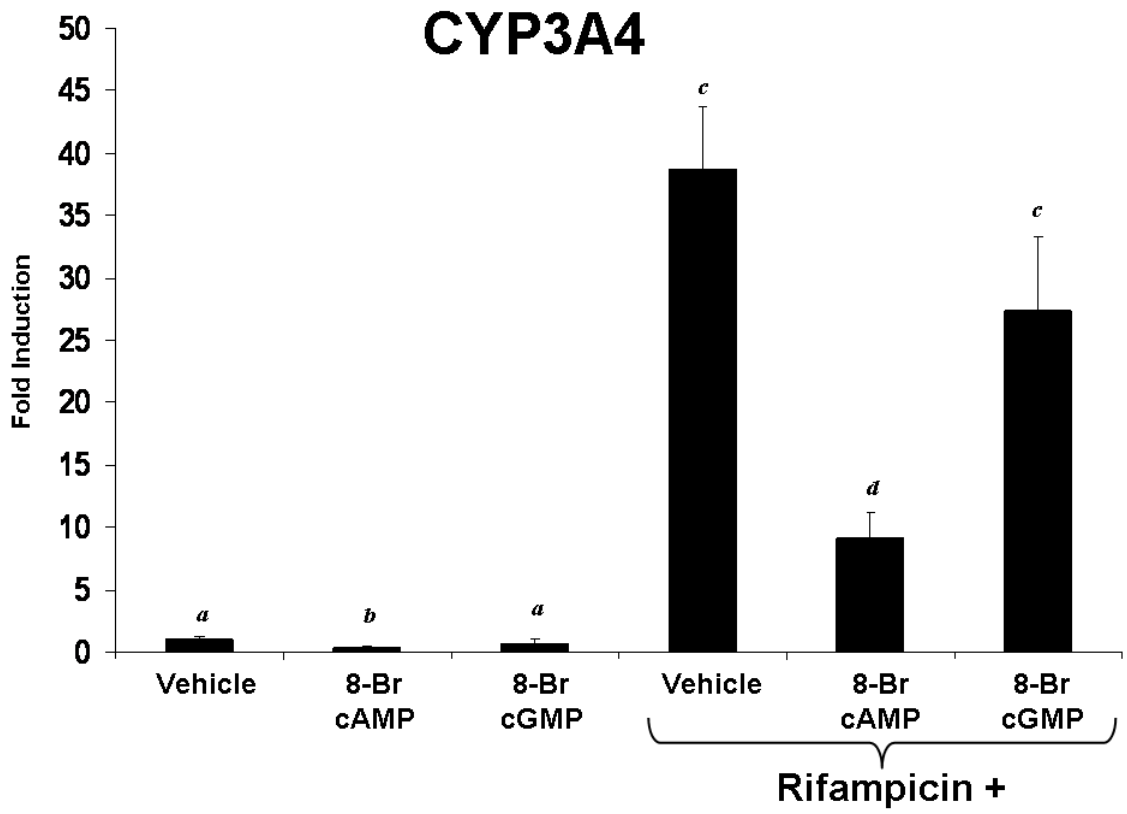


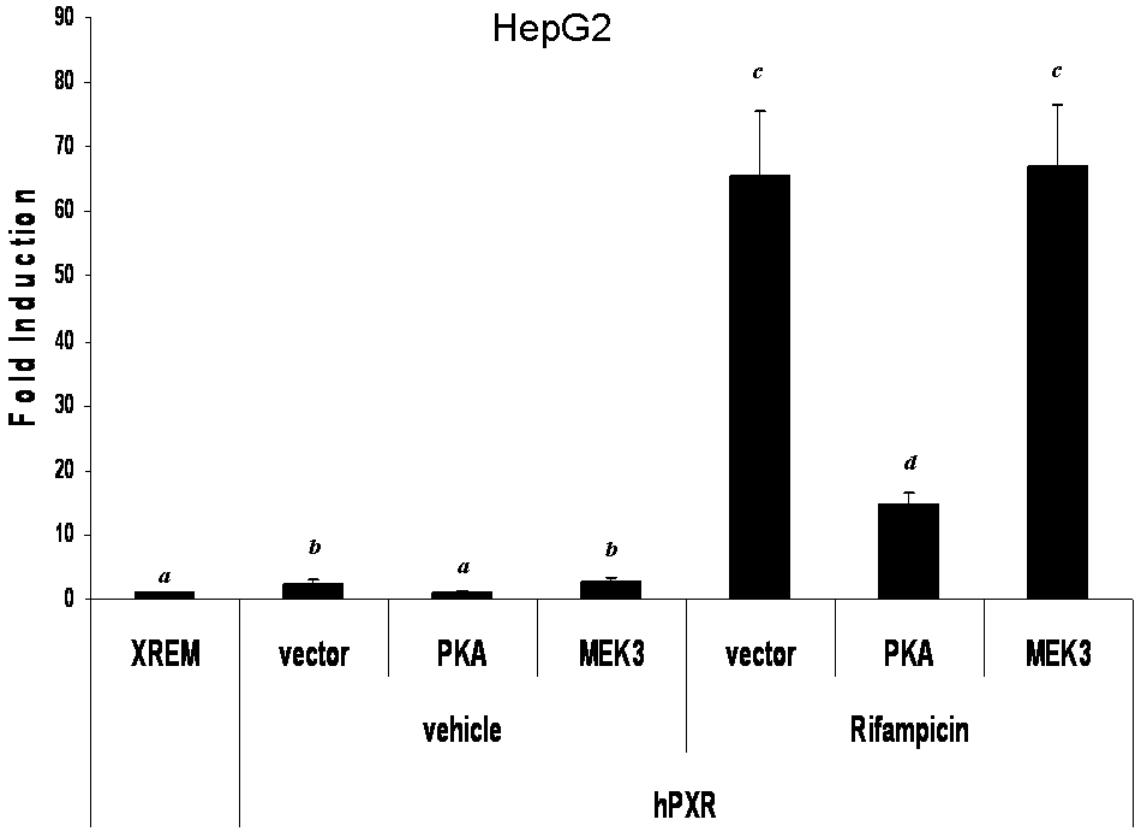
Figure 4-2. PKA activation has a species-specific effect on CYP3A gene expression in primary cultures of hepatocytes. Primary cultures of **(A)** mouse, **(B)** rat, and **(C)** human hepatocytes were treated with either 10μM PCN or 10 μM Rifampicin, and 1mM 8-Br-cAMP or 1mM 8-Br-cGMP for 24 hr and monitored for the induction of CYP3A orthologues. Data are expressed as fold induction over the vehicle control and are normalized to 18S expression and represent the mean of the replicates $\pm$ S.D. (n=3). Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

Species-specificity Resides in the Cyclic AMP Signaling Pathway. We next sought to determine whether the species-specific response to cAMP resides in the PXR protein, or is a function of how PKA signaling interfaces with PXR activity. The XREM-Luc reporter gene was used in HepG2 cells to determine if over-expression of PKA altered activity toward the *CYP3A4* promoter in a species-specific manner. A plasmid encoding human PXR was co-transfected with the XREM-Luc reporter gene in the presence and absence of an additional expression vector encoding the catalytic domain of PKA (Clontech). A plasmid encoding MEK3 (Clontech) was used as a control for kinase over-expression. Twenty-four hr post-transfection, selected wells were treated with rifampicin for an additional 24 hr (Figure 4-3A). As expected, rifampicin activated XREM-Luc reporter gene activity approximately 65-fold in the presence of human PXR. Over-expression of PKA repressed reporter gene activity by approximately 70%, while over-expression of MEK3 did not. Identical experiments using a plasmid encoding mouse PXR yielded similar results in that over-expression of PKA significantly repressed XREM-Luc reporter gene activity, while over-expression of MEK3 did not (Figure 4-3B). These data suggest that PKA signaling is a repressive signal in the human genetic background, and that repression of PXR activity is independent of the species of PXR protein.

We also wanted to determine whether human PXR would be positively regulated in the context of a murine hepatic genetic background. We therefore utilized our line of PXR knockout mice that have been engineered to express a human PXR transgene in liver [8]. Following liver perfusion, primary cultures of 'PXR-

humanized' mouse hepatocytes were treated with 8-Br-cAMP in the presence and absence of the human PXR agonist rifampicin (Figure 4-3C). As expected, treatment with rifampicin produced a 6-fold increase in *Cyp3a11* gene expression. Co-treatment of hepatocytes with 8-Br-cAMP and Rifampicin had a positive effect producing approximately 14-fold increase in *Cyp3a11* gene expression. As a negative control, treatment with 8-Br-cGMP had no effect on rifampicin-inducible gene expression. Taken together, these data further support the hypothesis that the observed species-specific interaction between cAMP and PXR activation in hepatocytes is a function of how PKA signaling interfaces with *CYP3A* gene expression across species, and is not due to differences in primary amino acid sequences in the human and mouse PXR proteins.

Figure 4-3A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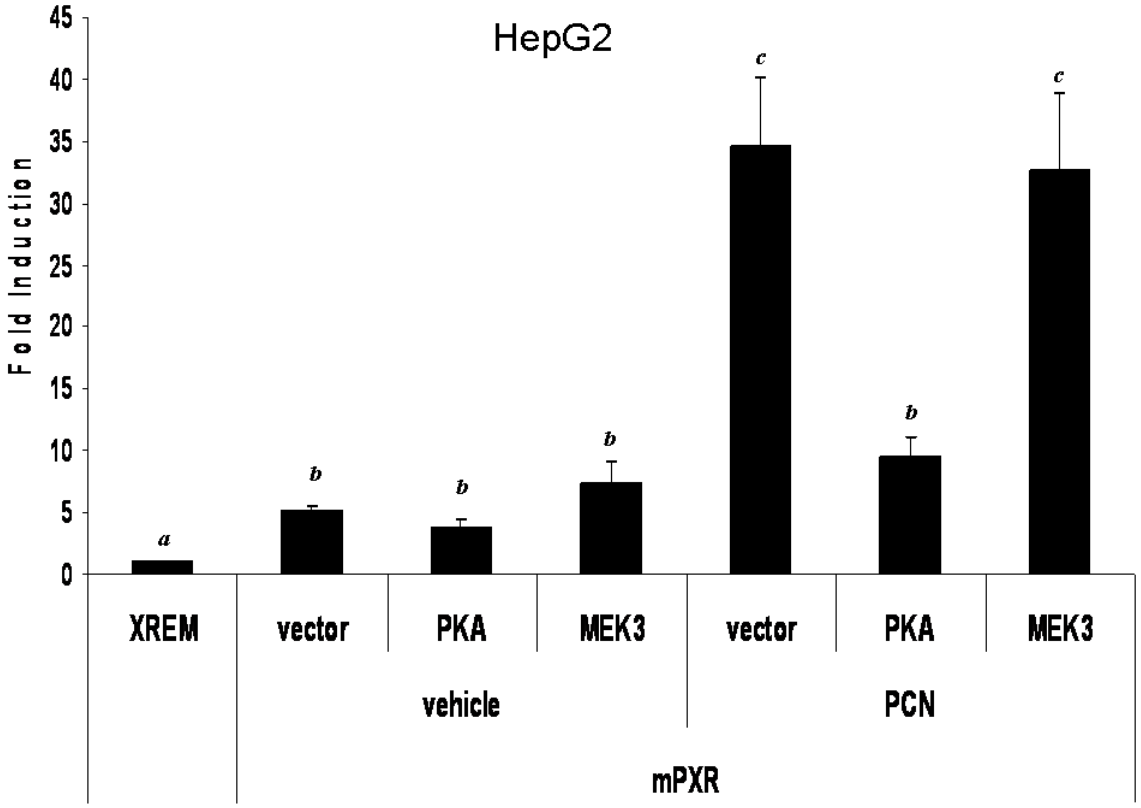
PKA
MEK3
vehicle
Rifampicin
hPXR

Figure 4-3B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mPXR

Figure 4-3C

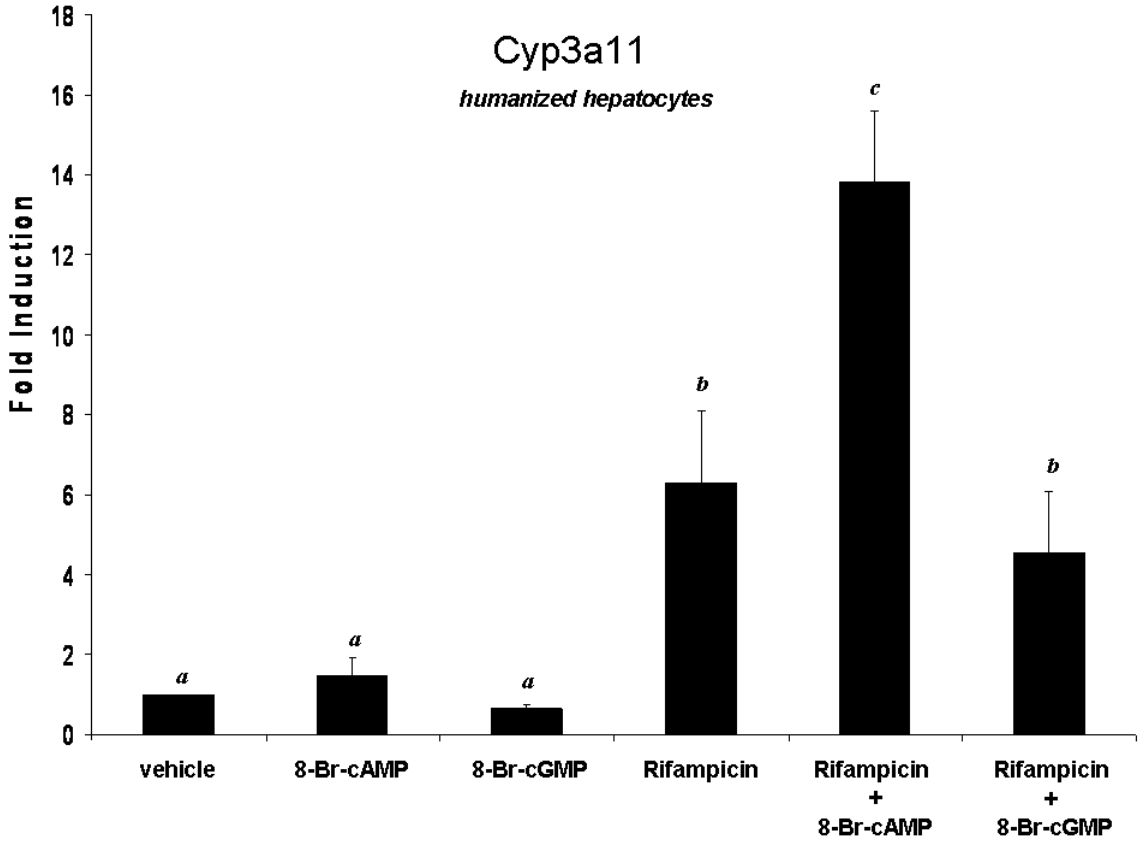
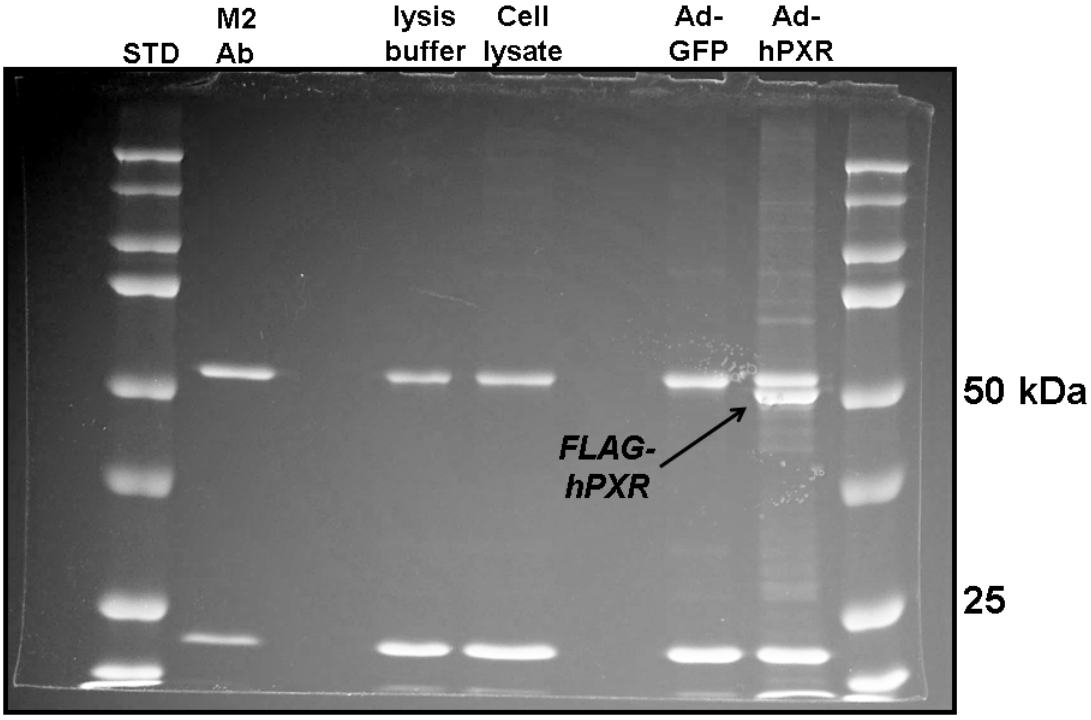


Figure 4-3. Species-specific modulation of PXR activity resides in the PKA signaling pathway. HepG2 cells were transfected with expression vectors for **(A)** human PXR or **(B)** mouse PXR together with the XREM-Luc reporter gene, in the presence or absence of an expression vector encoding constitutively active PKA. Cells were treated with 10 μM Rifampicin, 10 μM PCN, 1mM 8-Br-cAMP, and 1mM 8-Br-cGMP for 24 hours. Data are expressed as the mean of replicates $\pm$ S.D. (n=8) and are normalized to β-galactosidase activity. **(C)** Primary cultures of humanized mouse hepatocytes were treated with 10 μM Rifampicin in the presence and absence of 1mM 8-Br-cAMP and 1mM 8-Br-cGMP for 24 hours. Data are expressed as the mean of replicates + S.D. (n=3). Letters different from each other indicate a statistical difference between treatment groups (p<0.05).

Human PXR is a Phosphoprotein In Vitro and In Vivo. The extent to which down-regulation of human PXR activity is associated with alterations in the phosphorylation status of the PXR protein is unknown. We therefore created an adenoviral expression vector encoding a FLAG-tagged version of the human PXR protein in order to facilitate immuno-purification of the recombinant protein from cultured cells. Analysis of protein isolated from adenoviral-infected CV-1 cells using SDS-PAGE and coomassie-blue staining shows that the FLAG-hPXR protein is approximately 52 kDa (Figure 4-4A). Western-blot analysis using either anti-FLAG antibodies or anti-hPXR antibodies confirms that the recombinant protein is indeed FLAG-tagged human PXR (data not shown). When incubated *in vitro* with a series of catalytically active protein kinases, PXR served as the most effective substrate for PKA, followed by casein kinase II, glycogen synthase kinase, and protein kinase C (Figure 4-4B). Catalytically active AMP kinase and AKT2 were unable to directly phosphorylate the human PXR protein *in vitro* (data not shown).

Figure 4-4A



Coomassie Stained SDS-PAGE

Figure 4-4B

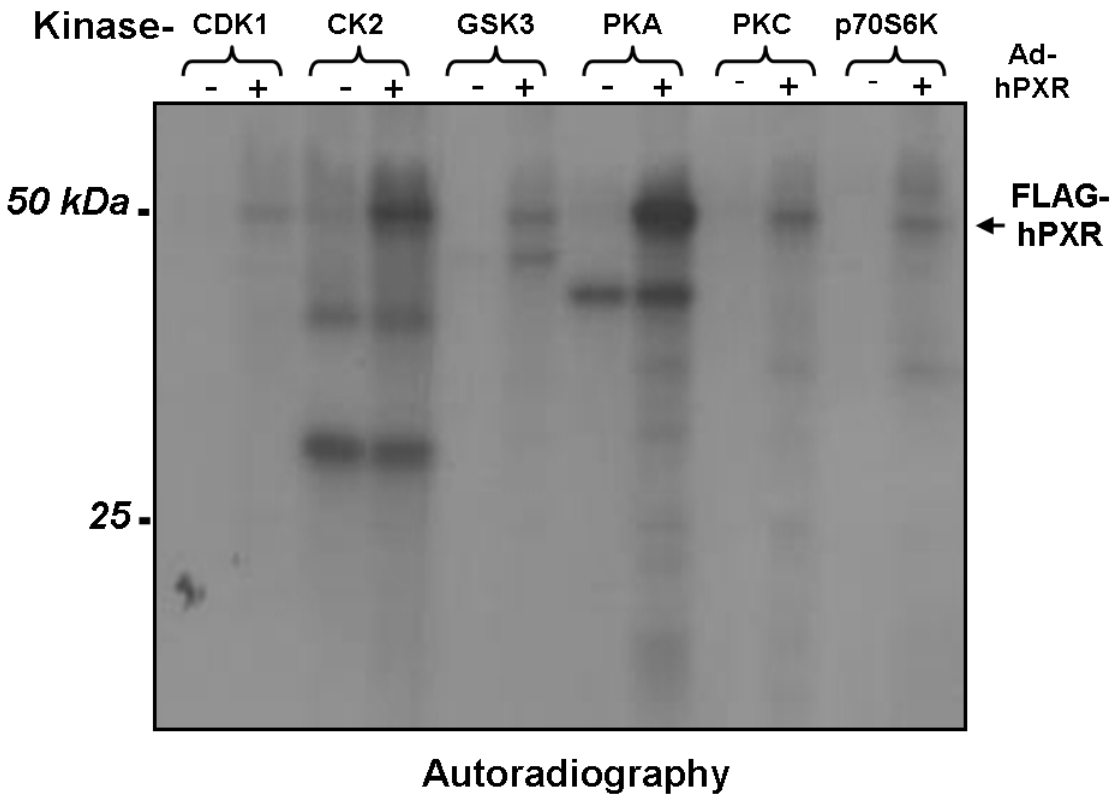


Figure 4-4. hPXR is phosphorylated by protein kinases *in vitro*. HepG2 cells were transduced with adenovirus expressing FLAG-tagged human PXR. **(A)** Human PXR was isolated from cellular extracts using immunoprecipitation with the M2 flag monoclonal antibody and detected using SDS-PAGE and coomassie staining. **(B)** The recombinant human PXR protein was phosphorylated with γ–^{32}P and myriad of kinases *in vitro*. Radiolabeled proteins were visualized by autoradiography.

To determine whether PKA can directly affect phosphorylation status of human PXR, CV-1 or HepG2 cells expressing recombinant FLAG-tagged human PXR protein were subjected to *in vivo* labeling using ^{32}P-orthophosphate and treated with either 8-Br-cAMP or 8-Br-cGMP in the presence and absence of rifampicin. The CV-1 cell line was chosen due to its ease of culturing as a confluent monolayer and uniform infection with the adenoviral vector; however identical results were obtained using either cell line. The human PXR protein was immunoprecipitated, resolved using SDS-PAGE, the gels were dried and subsequent autoradiography revealed that the human PXR exists as a phosphoprotein (Figure 4-5, *lane 4*). Immunoprecipitates from non-infected cells, blank-virus infected cells, and non-immune serum were included as negative controls (Figure 4-5, *lanes 1, 2, and 3*). The addition of rifampicin also had no effect upon the overall phosphorylation level of the human PXR protein (data not shown). A duplicate 'cold' experiment was performed and used for western-blot analysis with anti-hPXR antibodies to insure specificity of immunoprecipitation and roughly equivalent loading (Figure 4-5, *bottom panel*).

Figure 4-5

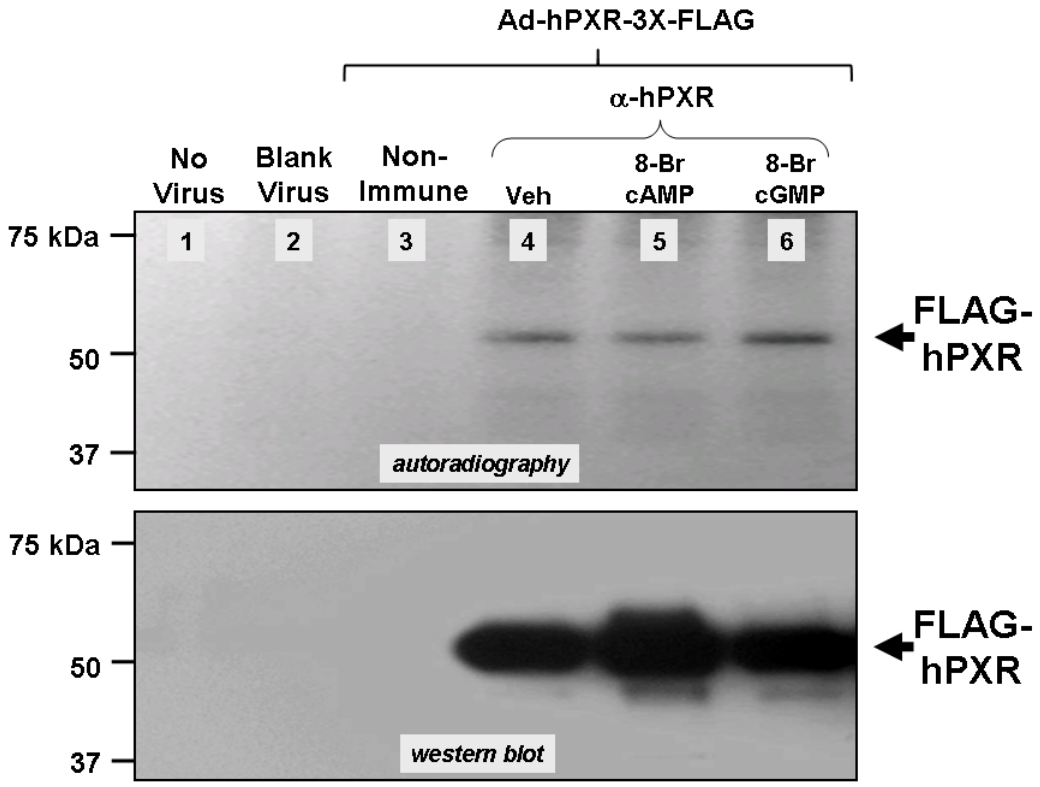


Figure 4-5. The human PXR protein exists as a phosphoprotein *in vivo*. HepG2 cells were transduced with adenoviral expression vector encoding FLAG-tagged human PXR. Cells were treated with phosphate-free medium for six hr and then treated with media containing 300 μCi/well ^{32}P-orthophosphate together with vehicle, 1mM 8-Br-cAMP, or 1mM 8-Br-cGMP for 14 hr. Phosphorylated PXR protein was visualized by autoradiography (upper panel). Total PXR protein was visualized by Western Blot using anti-HPXR antibodies in a duplicate cold experiment (lower panel).

We next screened a panel of seven commercially available monoclonal antibodies against the immuno-purified human PXR protein, which potentially recognize phosphoserine (four) and phosphothreonine (three) in the context of differing surrounding amino acid residues. Three of these antibodies, 1C8 (anti-phosphoserine), 14B3 and 1E11 (anti-phosphothreonine), effectively and specifically recognized immuno-purified human PXR phospho-protein in western blot analysis. To more closely examine whether activation of the PKA signaling pathway could alter the phosphorylation status of specific serine/threonine residues on the human PXR protein we treated adenoviral infected cells with either 8-Br-cAMP or 8-Br-cGMP in the presence or absence of rifampicin. Western blotting analysis revealed that treatment with 8-Br-cyclic AMP specifically up-regulated the recognized phosphothreonine content of the immunopurified human PXR protein, while treatment with 8-Br-cGMP did not (Figure 4-6, *lanes 4 and 5*). Treatment with rifampicin alone did not alter phosphothreonine content, while co-treatment with rifampicin prevented the up-regulation of 8-Br-cAMP-mediated phosphorylation of the human PXR protein (Figure 4-6, *lane 7*). Immunoprecipitates from cells infected with blank virus (Figure 4-6, *lane 1*), and immunopurified recombinant human PXR protein treated with lambda protein phosphatase (Figure 4-6, *lane 2*) were included as negative controls. The blot was stripped and re-probed with anti-FLAG monoclonal antibody to insure equal loading (Figure 4-6, *bottom panel*). Similar results were obtained with another monoclonal antibody anti-phosphothreonine- 1E11 (Calbiochem) (data not shown).

Figure 4-6

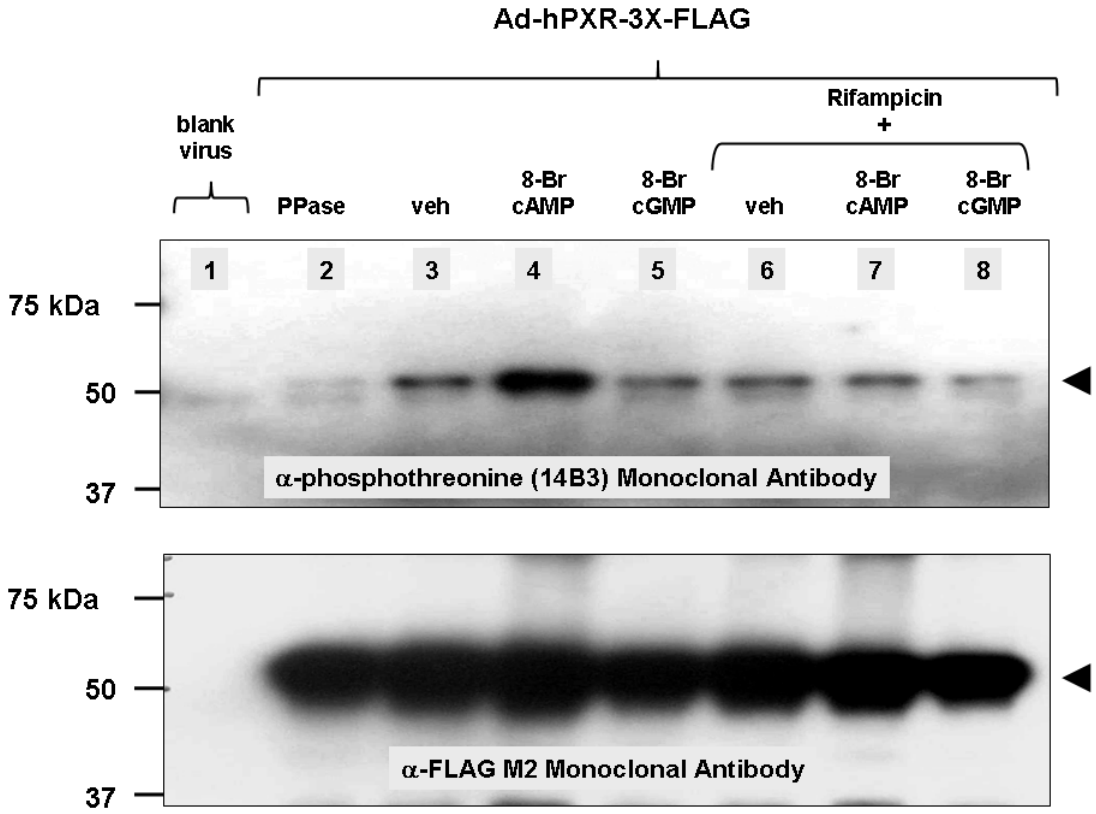
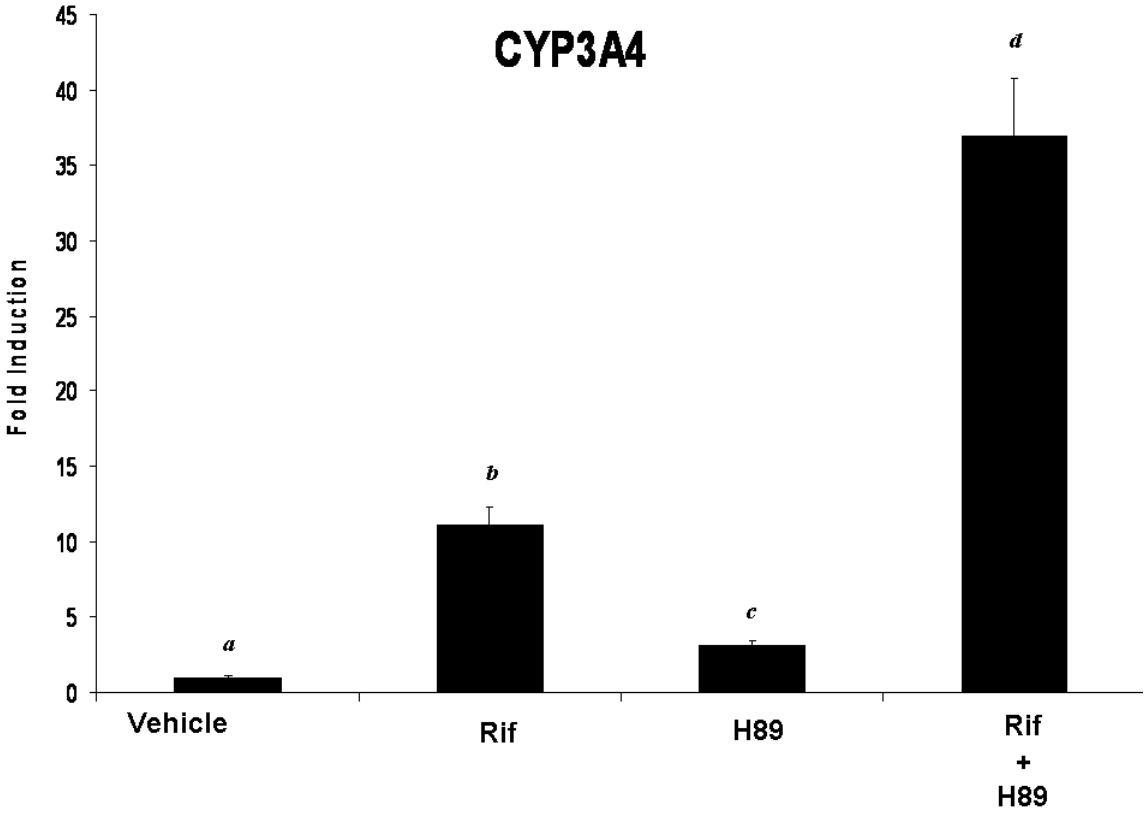


Figure 4-6. PKA signaling modulates the phosphorylation status of human PXR *in vivo*. CV-1 cells were transduced with an adenovirus expressing FLAG-tagged human PXR. Cells were treated with 10μM Rifampicin, 1mM 8Br-cAMP and 1mM 8Br-cGMP for 24 hours. The PXR protein was immunoprecipitated using anti-hPXR antibody and subjected to Western Blot analysis using anti-phosphothreonine 14B3 antibody (upper panel). The blot was stripped and re-probed with anti-FLAG monoclonal antibody to ensure equal loading (lower panel).

Physiological Levels of PKA Signaling Modulate PXR-target Gene Activation in a Species-specific Manner. Using pharmacological and biochemical methods to elevate PKA signaling, the experiments presented thus far are consistent with the hypothesis that PKA signaling interfaces with PXR activity in a species-specific manner. It was therefore of interest to determine whether endogenous PKA signaling modulates PXR-target gene activation across species. We therefore used the PKA-selective inhibitor H89 to examine the effect of inhibition of PKA on human and mouse PXR activity. HepG2 cells were transduced with the adenoviral expression vector encoding human PXR and treated with rifampicin in the presence and absence of H89 and expression levels of the *CYP3A4* gene were determined using Q-PCR (Figure 4-7A). Treatment of cells with rifampicin produced significant increases in *CYP3A4* gene expression. Treatment of cells with H89 alone produced a significant increase in the level of *CYP3A4*, although the levels were less than that produced by rifampicin treatment alone. Co-treatment of cells with rifampicin and H89 produced significantly increased levels of *CYP3A4* gene expression when compared to either treatment alone. The opposite trend was observed when similar experiments were conducted using primary cultures of mouse hepatocytes (Figure 4-7B). As expected, treatment with PCN alone produced significant increases in *Cyp3a11* gene expression levels, while treatment with H89 alone did not. Co-treatment of hepatocytes with PCN and H89 produced significantly lower levels of *Cyp3a11* gene expression when compared with that observed with PCN treatment

alone. Taken together, these data indicate that physiological levels of cyclic AMP and PKA signaling interface with PXR activity in a species-specific manner.

Figure 4-7A


CYP3A4
Fold Induction
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40
35
30
25
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b
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Rif
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+
H89

Figure 4-7B

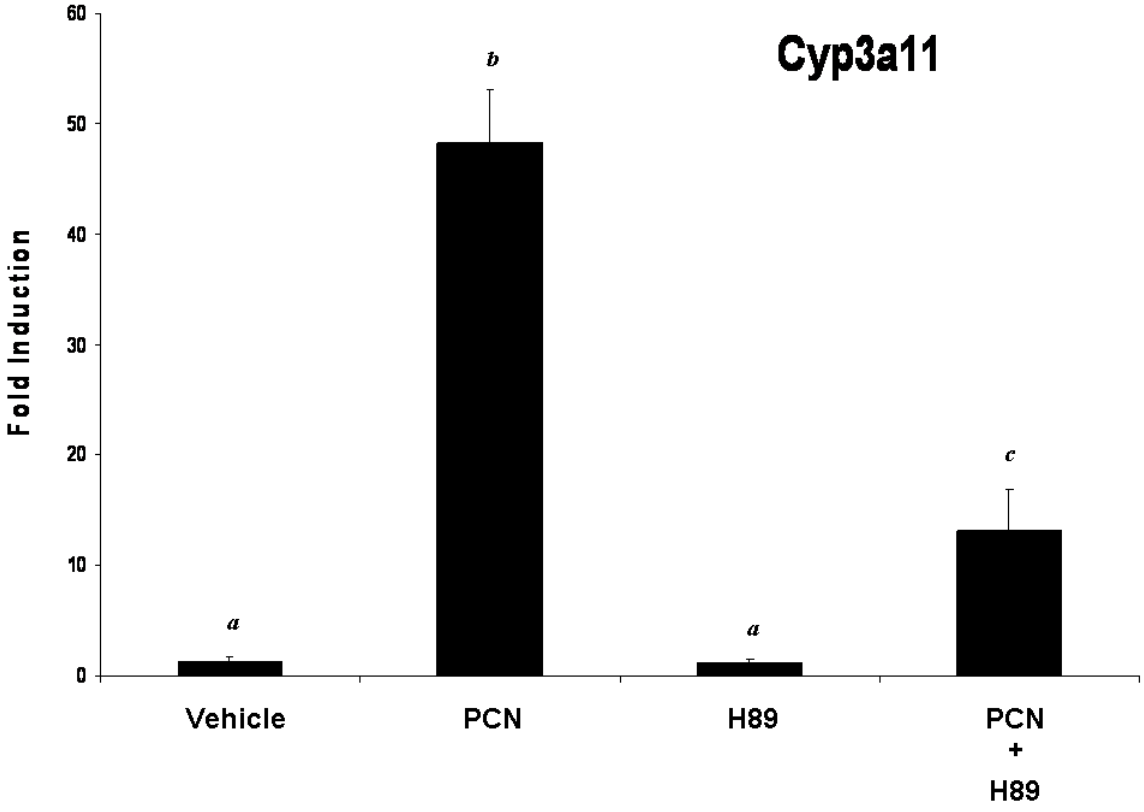

Figure 4-7. Endogenous levels of PKA signaling modulate PXR activity in a species-specific manner. (A) HepG2 cells were transduced with the adenoviral expression vector encoding FLAG-tagged human PXR. Cells were treated with vehicle or 10 μM rifampicin, 10 μM H89, or 10 μM rifampicin plus 10 μM H89. **(B)** Primary cultures of mouse hepatocytes were treated with vehicle or 10 μM rifampicin, 10 μM H89, or 10 μM rifampicin plus 10 μM H89. Endogenous levels of *CYP3A* were determined using Q-PCR analysis. Data are expressed as the mean of replicates + S.D. (n=3). Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

Activation of PKA Signaling Promotes Association of Human PXR with NCoR. Because rifampicin-activated human PXR localized in part to the nucleus, and was repressed by both treatment with 8-Br-cAMP and co-expression of the catalytic subunit of PKA, we reasoned that PKA signaling might modulate PXR's ability to interact with nuclear receptor corepressor protein (NCoR). To investigate this possibility we used the nuclear receptor interaction domain (Arg 2065 to Gly 2287) from NCoR (GAL4-NCoR) and full-length human PXR fused to VP16 (VP16-hPXR) in the mammalian two-hybrid system. Transfection of CV-1 cells with GAL4-NCoR together with VP16-human PXR produced increased reporter gene activity, while transfection of GAL4-NCoR alone did not (Figure 4-8). As expected, treatment of transfected cells with 10 μM rifampicin decreased the strength of interaction between these two fusion proteins. Co-transfection of the catalytic subunit of PKA increased the strength of association between PXR and NCoR by approximately five-fold, whereas co-transfection of the constitutively active kinase MEK1 had no effect. Interestingly, while administration of rifampicin in the presence of PKA weakened the association between NCoR and human PXR, the relative strength of association was well above that obtained in the absence of PKA. Similar results were obtained when using 8-Br-cAMP to activate PKA signaling and 8-Br-cGMP as a negative control in HepG2 cells (data not shown).

Figure 4-8

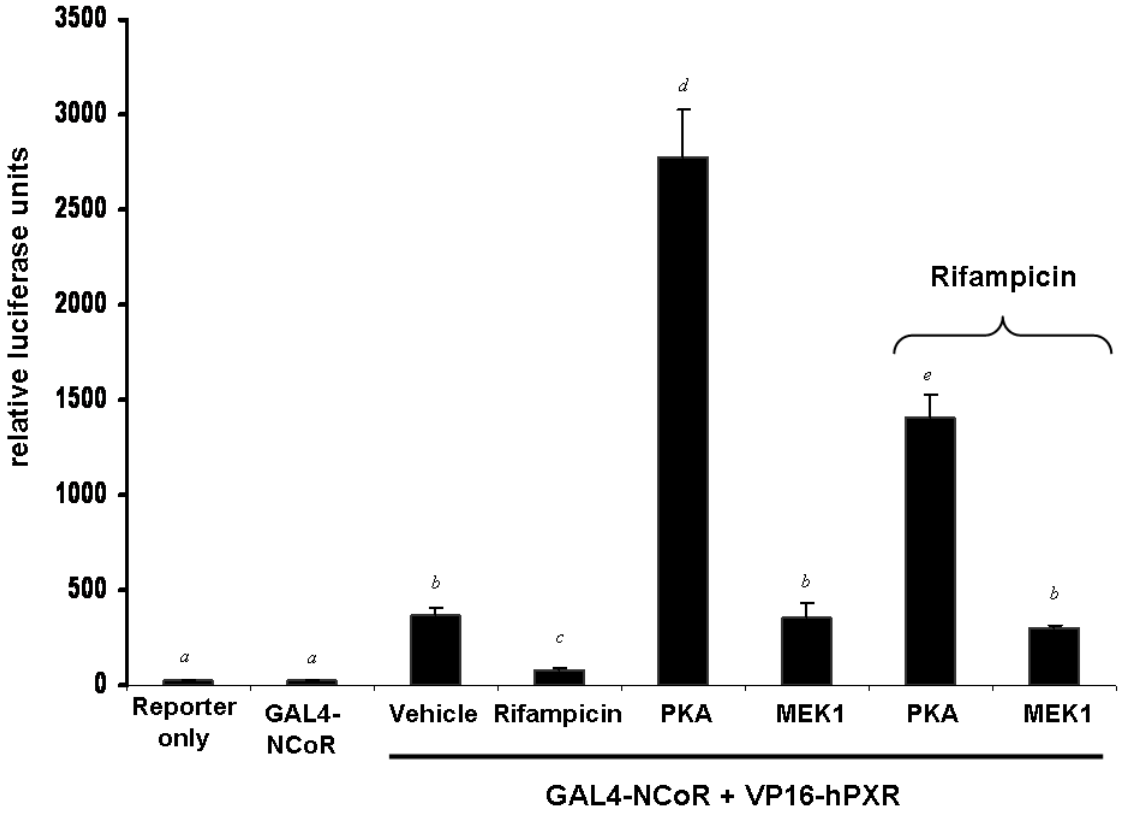

Figure 4-8. PKA increases the strength of interaction between hPXR and NCoR in mammalian-2-hybrid reporter gene assays. CV-1 cells were transfected with expression vectors for Gal4-NCoR receptor interacting domain, VP16-human PXR, pFR-Luc reporter gene, and constitutively active kinases. Cells were treated with 10 μM Rifampicin for 24 hours. Data are expressed as the mean of replicates + S.D. (n=8) and are normalized to β-galactosidase activity. Letters different from each other indicate a statistical difference between treatment groups (p<0.05).

4.4 Discussion

Diabetes, fasting, obesity, protein-calorie malnutrition and long-term alcohol consumption all produce alterations in the expression and activity of hepatic drug metabolizing enzymes [23]. Moreover, the expression and activity of CYP3A4, the primary drug metabolizing enzyme found in liver and intestine, is rapidly and dramatically repressed in response to acute inflammatory states [24]. Because PXR is a master-regulator of drug-inducible transcription of the *CYP3A4* gene, there is a high level of interest in understanding the potential role of this transcription factor in mediating transcriptional suppression during these specific pathological conditions. While much is known regarding the identity of target genes and ligands for this important nuclear receptor protein, comparatively little is known regarding the post-translational modification of the human PXR protein. Therefore, we and others have sought to understand the molecular mechanisms that comprise the potential interface between human PXR and important cellular signal transduction cascades that mediate repression of drug metabolism, energy metabolism, and glucose production in liver.

The species-specific nature of nuclear receptor signaling is a well documented and highly relevant area of scientific inquiry. For example, although peroxisome proliferators have carcinogenic consequences in the livers of rodents, epidemiological studies have revealed that similar effects are unlikely to occur in humans [25]. Additionally, PB has been used for decades as the prototypical non-genotoxic tumor promoting agent in numerous rodent studies of hepatocarcinogenesis, however, epidemiological studies indicate that PB does not cause liver tumors in humans [26].

Although the primary event governing activation of nuclear receptors is ligand-binding, increasing amounts of evidence suggest that cell signaling pathways and modulation of nuclear receptor-cofactor-phosphorylation status also determines overall responsiveness to environmental stimuli [27, 28]. Phosphorylation has been implicated in regulation of (1) nuclear receptor transactivation capacity, (2) DNA-binding, (3) sub-cellular localization, (4) protein cofactor interaction profile, and (5) protein stability.

It is a long-standing observation that treatment of patients with rifampicin tends to suppress immunological responses in liver cells. The precise molecular basis for the repression of the inflammatory response following PXR activation is not currently known, although it likely involves a kinase-mediated signaling cascade. Symmetrically, activation of the inflammatory response by treatment with lipopolysaccharide or tumor necrosis factor α decreases PXR-mediated gene activation. Recent studies suggest that activation of NF-κB interferes with the formation of the PXR-RXR heterodimeric complex on the *CYP3A4* promoter [29]. Additional studies in rodents suggest that down-regulation of PXR-target genes by inflammatory cytokines is PXR-dependent [30]. Our results provide additional evidence for a key interface between kinase-mediated signal transduction pathways and PXR activity. Moreover, our results provide compelling evidence for pronounced species-specific differences in the coupling of pivotal kinase cascades and PXR activity. Moreover, since PKA signaling is up-regulated during acute

inflammation, our results describe a potential molecular mechanism for the observed repression of PXR-target gene expression during this pathophysiological condition.

Recent research indicates that some metabolic signal transduction pathways interface with PXR. The extent to which this interaction is dependent on kinase signaling and the phosphorylation status of PXR is unknown and requires further investigation. Activation of PXR has recently been shown to decrease energy metabolism and increase hepatic triglyceride levels through down-regulation of gluconeogenesis, fatty acid oxidation, and ketogenesis and by up-regulating hepatic lipogenesis [31-35]. The crosstalk of PXR with these fundamental biological processes is thought to be due to PXR's ability to interact directly with FoxO1, FoxA2, CREB, and PGC-1α. In addition, a recent study indicates that human PXR can be phosphorylated at more than one site by the serine-threonine protein kinase CDK2 *in vitro* [36]. The same study showed that PXR activity toward the *CYP3A4* promoter is inhibited during S-phase of the cell cycle.

In the current study we show that activity of the PXR protein is modulated in hepatocytes by treatment with 8-Br-cAMP, a well characterized and specific activator of PKA in a species-specific manner. We also show that the phosphorylation status of threonine residues is altered following activation of PKA signaling in cultured cell lines. There are several important implications of these findings. First, this is the first demonstration that we are aware of that PXR exists in cells as a phosphoprotein. Second, the demonstrated alterations in PXR's threonine phosphorylation status and modulation of NCoR corepressor protein recruitment following activation of PKA

signaling impart a new level of understanding regarding the potential molecular basis of repression of PXR activity by various signal transduction pathways. Specifically, our data are consistent with the model that phosphorylation of the human PXR protein, or PXR-associated protein, favors recruitment of corepressor multi-protein complexes thereby producing repression of drug-inducible PXR-target gene expression. However, it should be noted here that our data do not exclude the possibility that the molecular basis of PKA-mediated repression of PXR activity is multi-factorial in nature. For example, alterations in the phosphorylation status of the PXR protein could in principle inhibit PXR's DNA binding capacity, alter its subcellular distribution, reduce PXR protein stability, as well as prevent PXR association with coactivator proteins. Whether repression of PXR activity by PKA signaling is regulated by direct phosphorylation of the human PXR protein is not currently known, however, extensive mutagenesis of the human PXR protein failed to identify a single residue that was responsible for PKA-dependent repression in the reporter gene assay (data not shown). While our western blotting data generated using an anti-phosphothreonine antibody suggests that the level of threonine phosphorylation is altered by cAMP in the recombinant human PXR, the question remains as to how PKA signaling represses PXR activity in the presence of rifampicin. Our western blot data should be interpreted carefully since the anti-phospho antibodies used here recognize only a subset of phosphothreonine residues in any given protein due to the nature of the surrounding amino acid sequences and the accessibility of the given epitope. Finally, our description of the species-specific

effects of PKA signaling raises the possibility that pre-clinical testing of novel drug candidates in 'humanized' PXR and CAR mice poses more of a problem than previously realized. Future experiments should be focused upon determining which serine and/or threonine residues are subject to regulated phosphorylation and which kinases and phosphatases alter the activity of the human PXR protein.

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Chapter 5: Phosphomimetic Mutation of Potential Phosphorylation Sites within the PXR Protein Modulates PXR Activity

5.1 Introduction

Nuclear receptors (NRs) comprise a large group of transcription factors, with 48 members present in the human genome, which control diverse biological functions including metabolism, homeostasis, reproduction and development. All members of the NR super-family share several conserved structural domains that are essential for receptor function. The C-terminal region contains a ligand binding domain (LBD) and a ligand-dependent activation function-2 (AF-2). The LBD is connected to the DNA binding domain (DBD) and an N-terminal activation function-1 (AF-1) by a hinge region (H) [1]. Most NRs are conventionally activated by the binding of small lipophilic ligands such as hormones, fatty acids, oxysterols, bile acids, and xenobiotics [2]. In addition to ligand binding, numerous studies have implicated signaling pathways in the modulation of NR activities. Many liver-enriched NRs are targets of phosphorylation, a post-translational modification and regulatory mechanism, which not only is critical for protein functions, but also enables cross-talk between diverse signaling pathways [3, 4]. NRs can be phosphorylated constitutively, in response to ligand, or in response to the activation of various signaling pathways. NRs can be phosphorylated in any of their conserved domains. In fact, multiple aspects of NR activity are modulated when specific sites within the receptor are phosphorylated including transcriptional activity, protein expression and stability, sub-cellular localization, heterodimerization, and cofactor binding.

In comparison to other NRs, we have a meager understanding regarding how the NR pregnane x receptor (PXR) is regulated by phosphorylation. PXR is a promiscuous receptor activated by a wide range of compounds including steroids, bile acids, and variety of drugs and natural compounds. PXR has been characterized as a master regulator of xenobiotic-inducible cytochrome-p450 (CYP) gene expression in liver. In fact, the CYPs identified as PXR target genes are responsible for the oxidative metabolism of more than 60% of clinically prescribed drugs. Furthermore, it is now clear that PXR regulates the activation of a group of genes that encode CYPs, additional drug metabolizing enzymes, and drug transporter proteins [5-7]. In this manner, PXR activation increases metabolism, transport and elimination of potentially toxic compounds from the body and represents the basis for an important class of drug interactions. In addition, recent evidence suggests a role for PXR in hepatic glucose and lipid metabolism [8, 9], endocrine homeostasis [10, 11], inflammation [12-14], and drug resistance [15, 16].

It has been known for a number of years that drug-inducible CYP gene expression is responsive to kinase signaling pathways; however, the exact mechanism by which these pathways intersect with PXR is unknown. Activation of cyclic AMP-dependent protein kinase (PKA) signaling has been shown to modulate PXR activity [17]. While PKA activation potentiates the drug-inducible expression of *Cyp3a11* in mouse hepatocytes, it serves as a repressive signal in both human and rat hepatocytes. Kinase assays show that PXR can serve as a substrate for PKA *in vitro*. It has also been shown that PXR exists as a phospho-protein *in vivo* and that its phospho-

threonine status is modulated by the activation of PKA signaling [18]. This evidence suggests one potential mechanism for PKA-mediated modulation of *CYP3A* gene expression. In addition, the activation of protein kinase C (PKC) signaling has been shown to repress PXR activity by increasing the strength of interaction between PXR and the co-repressor NCoR, and by abolishing the ligand-dependent interaction between PXR and SRC-1 [19]. Cyclin-dependent kinase 2 (Cdk2) also attenuates the activation of *CYP3A4* gene expression. PXR is a suitable substrate for the Cdk2 enzyme *in vitro*, and a phosphomimetic mutation at a putative Cdk phosphorylation site at (S350D) appears to impair the function of hPXR, whereas a phosphorylation-deficient mutation (S350A) conferred resistance to the repressive effects of Cdk2 on a reporter gene in HepG2 cells [20]. An additional study has identified a phosphomimetic mutation within the DBD (T57D) that is associated with the loss of function of hPXR. Furthermore, PXR was identified as a substrate for p70 S6 kinase *in vitro* and the phosphorylation-deficient mutation (T57A) conferred resistance to the inhibitory effect of p70 S6K [21]. The results of these studies suggest that the activity of PXR is modulated by changes in phosphorylation. However, the direct phosphorylation and subsequent modulation of PXR activity has not been demonstrated *in vivo*.

Understanding the mechanisms that regulate the expression of drug metabolizing enzymes is critical in the development of effective clinical therapeutic strategies and to avoid potentially dangerous drug interactions. Since the activity of PXR is noticeably regulated by the activation of kinase signaling pathways, an

understanding of the phosphorylation dependent events in PXR signaling is necessary for safe and effective and drug design and clinical use. To date, there are no studies that we are aware of that have systematically investigated the potential phosphorylation sites within the PXR protein in regard to PXR activity. In this study, we identify 18 potential phosphorylation sites throughout the hPXR protein by either kinase consensus site prediction or by sequence homology of known phosphorylation sites within other NRs. Using a site-directed mutagenesis based approach; we identified 6 sites of interest at S8, T57, S208, S305, S350, and T408 that warranted further characterization. A phosphomimetic mutation (Asp) at these 6 sites decreases the basal activity of PXR in a cell based reporter gene assay, whereas phosphorylation-deficient mutation (Ala) results in either no change or in an increase in PXR activity. Phosphomimetic mutations at T57 and T408 further abolish the ligand-inducible transactivation of PXR on the XREM reporter gene. Gel mobility shift assay experiments reveal that phosphomimetic mutation at T57, located within the DBD, abolishes the ability of PXR to bind to its response elements whereas the phosphorylation-deficient mutation does not. Furthermore, phosphomimetic mutations at consensus sites within the LBD at S305, S350, and T408 decrease the ability of PXR to heterodimerize with its partner RXRα. Mammalian 2-hybrid experiments reveal that phosphomimetic mutations at S208 and S305 increase the strength of interaction between PXR and the co-repressor NCoR and decrease the strength of interaction between PXR and the co-activator SRC-2. Taken together, these data suggest that PXR may potentially be regulated by phosphorylation at

specific amino acid residues. Furthermore, phosphorylation at specific residues can uniquely regulate PXR activity by altering either one or a combination of the following parameters: transactivation capacity, DNA-binding, heterodimerization, and co-factor binding.

5.2 Materials and Methods

Compounds and Plasmids. Unless otherwise stated, all chemical compounds were purchased from Sigma-Aldrich. The pSG5-hPXR, GAL4-SRC1, GAL4-SRC2, GAL4-PBP, and GAL4-NCoR1 expression vectors were described previously [19]. The pFR-LUC reporter gene, which is responsive to GAL4-fusion proteins, is commercially available (BD Biosciences). The pCMX-flag RXRα expression vector was a kind gift from Dr. Koren Mann. PXR wild-type and mutant constructs were fused to the VP16 transcriptional activation domain by sub-cloning into the pVP16 expression vector (Clontech) at EcoRI and BamHI restriction sites.

Site-directed mutagenesis. Consensus serine and threonine phosphorylation sites within the human PXR protein were identified using the NetPhos 2.0 server. Eighteen potential phosphorylation sites were mutated to an aspartic acid, a phosphomimetic mutation, and an alanine, a non-phosphomimetic mutation. The mutant pSG5-hPXR expression vectors were generated by site-directed mutagenesis using the QuickChange Mutagenesis system (Stratagene). Primer sequences used for site-directed mutagenesis are shown in Table 5-1.

Table 5-1. Oligo sequences for site-directed mutagenesis of the human PXR protein.

Amino Acid	Oligos for mutagenesis to D
S8	5’ ggaggtgagacccaaagaagactggaaccatgctg 3’ cagcatggttccagtcttctttgggtctcacctcc
T20	5’ gactttgtacactgtgaggacgatgagtctgttcctggaaagccc 3’ gggctttccaggaacagactcatcgtcctcacagtgtacaaagtc
T57	5’ ctggctatcacttcaatgtcatggattgtgaaggatgcaagggcttttt 3’ aaaaagcccttgcatccttcacaatccatgacattgaagtgatagccag
T90	5’ agatcacccggaaggaccggcgacagtgcc 3’ ggcactgtcgccggtccttccgggtgatct
S105	5’ gcgcaagtgcctggaggacggcatgaagaaggag 3’ ctccttcttcatgccgtcctccaggcacttgcgc
S114	5’ gaaggagatgatcatggacgacgaggccgtggag 3’ ctccacggcctcgtcgtccatgatcatctccttc
S130	5’ cttgatcaagcggaagaaagacgaacggacagggactcagc 3’ gctgagtccctgtccgttcgtctttcttccgcttgatcaag
T133	5’ cggaagaaaagtgaacgggatgggactcagccactggga 3’ tcccagtggctgagtcccatcccgttcacttttcttccg
T135	5’ aagtgaacggacaggggatcagccactgggagtg 3’ cactcccagtggctgatcccctgtccgttcactt
S180	5’ ccaggggtgcttagcgatggctgcgagttgcc 3’ ggcaactcgcagccatcgctaagcacccctgg
S192	5’ cagagtctctgcaggccccagatagggaagaagctgcc 3’ ggcagcttcttccctatctggggcctgcagagactctg
S208	5’ ggtccggaaagatctgtgcgatttgaaggtctctctgcag 3’ ctgcagagagaccttcaaatcgcacagatctttccggacc
S231	5’ caaacccccagccgacgatggcgggaaagagatc 3’ gatctctttcccgccatcgtcggctgggggtttg
S274	5’ atcgaggaccagatcgacctgctgaaggggggc 3’ gccccccttcagcaggtcgatctggtcctcgat
T290	5’ ctgtgtcaactgagattcaacgatgtgttcaacgcggagactgga 3’ tccagtctccgcgttgaacacatcgttgaatctcagttgacacag
S305	5’ ggagtgtggccggctggactactgcttggaagac 3’ gtcttccaagcagtagtccagccggccacactcc
S350	5’ ccatctccctcttcgacccagaccgcccag 3’ ctgggcggtctgggtcgaagagggagatgg
T408	5’ atcaatgctcagcacgaccagcggctgctgcg 3’ cgcagcagccgctggtcgtgctgagcattgat

Amino Acid	Oligos for mutagenesis to A
S8	5’ ggaggtgagacccaaagaagcctggaaccatgctg 3’ cagcatggttccaggcttctttgggtctcacctcc
T20	5’ tgtacactgtgaggacgcagagtctgttcctgg 3’ ccaggaacagactctgcgtcctcacagtgtaca
T57	5’ ctatcacttcaatgtcatggcatgtgaaggatgcaaggg 3’ cccttgcatccttcacatgccatgacattgaagtgatag
T90	5’ gatcacccggaaggcccggcgacagtg 3’ cactgtcgccgggccttccgggtgatc
S105	5’ cgcaagtgcctggaggccggcatgaagaagga 3’ tccttcttcatgccggcctccaggcacttgcg
S114	5’ gaaggagatgatcatggccgacgaggccgtg 3’ cacggcctcgtcggccatgatcatctccttc
S130	5’ cttgatcaagcggaagaaagctgaacggacagggactcagc 3’ gctgagtccctgtccgttcagctttcttccgcttgatcaag
T133	5’ gaagaaaagtgaacgggcagggactcagccact 3’ agtggctgagtccctgcccgttcacttttcttc
T135	5’ tgaacggacaggggctcagccactggg 3’ cccagtggctgagcccctgtccgttca
S180	5’ caggggtgcttagcgctggctgcgagttgc 3’ gcaactcgcagccagcgctaagcacccctg
S192	5’ ctctgcaggccccagcgagggaagaag 3’ cttcttccctcgctggggcctgcagag
S208	5’ tccggaaagatctgtgcgctttgaaggtctctctg 3’ cagagagaccttcaaagcgcacagatctttccgga
S231	5’ acccccagccgacgctggcgggaaagag 3’ ctctttcccgccagcgtcggctgggggt
S274	5’ cgaggaccagatcgccctgctgaaggg 3’ cccttcagcagggcgatctggtcctcg
T290	5’ gtcaactgagattcaacgcagtgttcaacgcggag 3’ ctccgcgttgaacactgcgttgaatctcagttgac
S305	5’ gtgtggccggctggcctactgcttgga 3’ tccaagcagtaggccagccggccacac
S350	5’ catctccctcttcgccccagaccgccc 3’ gggcggtctggggcgaagagggagatg
T408	5’ caatgctcagcacgcccagcggctgct 3’ agcagccgctgggcgtgctgagcattg

Transient Transfection and Reporter Gene Analysis. The XREM-LUC reporter gene assays were and the mammalian two-hybrid system assays were performed as described previously [19, 22]. Briefly, CV-1 cells were plated in 96-well plates at a density of 7000 cells per well. After 24 hours the cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. The PXR transactivation assays were transfected with 110 ng of DNA per well containing SV40-βgal (40ng), XREM-Luc (20ng), pSG5-hPXR (5ng), and pBluescript (45ng). The mammalian two-hybrid assays were transfected with 110 ng of DNA per well containing SV40-βgal (40ng), pFR-Luc (20ng), Gal4-Cofactor (20ng), VP16-hPXR (10ng), and pBluescript (20ng). The next day the cells were drug-treated for 24 hours with vehicle or 10 μM rifampicin. Luciferase and β-galactosidase activities were determined using a standard luciferase assay system (Promega).

Gel Shift Assay. Wild-type and mutant human PXR and RXRα were synthesized in vitro using the TNT reticulocyte lysate system (Promega) according to the manufacturer's instructions. Each 20μl gel mobility shift reaction contained 10mM Tris pH 8, 60mM KCl, 0.1% NP-40, 6% glycerol, 2mM DTT, 2μg poly-dIdC, and 5μl total in vitro translated protein. Competitor oligonucleotides were added in 5 and 50 fold molar excess. A monoclonal antibody for hPXR (Santa Cruz) was added to visualize a super-shift. After incubation on ice for 10 minutes 4ng of ^{32}P labeled oligonucleotide was added. After an additional 10 minute incubation on ice, the DNA-protein complexes were resolved on a 4% polyacrylamide gel. The gel was

dried and subjected to autoradiography. The following double stranded oligonucleotides were used as radio-labeled probes or cold competitors as indicated: CYP3A4 ER6 (5'-gAT CAA TAT gAA CTC AAA ggA ggT CAg Tg) and mutated CYP3A4 ER6 (5'-gAT CAA TAT gTT CTC AAA ggA gAA CAg Tg).

PXR Heterodimerization Assay. Wild-type and mutant human PXR and flag-RXRα were synthesized in vitro using the TNT reticulocyte lysate system (Promega). Ten μl of each *in vitro* translated protein was diluted to 500 μl in immunoprecipitation buffer (PBS, 0.5% NP-40, and protease inhibitors). The lysates were pre-cleared with 20 μl of immobilized protein A (Repligen). Flag-tagged RXRα was immunoprecipitated using anti-flag M2 affinity gel (Sigma-Aldrich) or non-immune IgG as indicated. Free immune complexes were captured and washed three times with lysis buffer. Immunoprecipitated protein complexes were subjected to SDS-PAGE and transferred to PVDF membranes (Millipore). Western Blot analysis was performed using a polyclonal antibody generated to detect the human PXR protein.

5.3 Results

Identification and mutation of predicted phosphorylation sites within the PXR protein. Potential serine and threonine phosphorylation sites within the PXR protein were identified using the NetPhos 2.0 Server. Forty-eight potential serine and threonine phosphorylation sites were identified and scored from 0.000-1.000 based on the likelihood of phosphorylation (Figure 5-1A). The serine and threonine residues that have higher scores are indicated as likely phosphorylation sites. In this study,

serine and threonine residues that were scored above the threshold of 0.500 were selected for mutagenesis (Figure 5-1B). In addition to the residues that were scored above 0.500, we also selected T57, T133, and S274 for mutagenesis. T57 has been previously characterized as a PXR phosphorylation site that is potentially regulated by p70 S6K [21]. Preliminary mass spectroscopy data from our lab has indicated T133 as a potential PXR phosphorylation site (data not shown). Finally, S274 was chosen due to its location within a conserved nuclear localization signal. Therefore, eighteen residues in total were mutated to either a negatively charged phosphomimetic residue (Asp) or a phospho-deficient residue containing a hydrophobic side chain (Ala). These 18 residues are located throughout the conserved NR domains of the PXR protein. It is also noteworthy that three of the residues S180, S192, and S208 are located in a portion of the PXR protein that is deleted in the alternative splice variant hPXR.2 (Figure 5-1C).

Figure 5-1A

Threonine predictions

Position	Context	Score	
20	HCEDTESVP	0.825	*T*
49	GDKATGYHF	0.064	.
57	FNVMTCEGC	0.042	.
87	ACEITRKTR	0.167	.
90	ITRKTRRQC	0.949	*T*
133	KSERTGTQP	0.145	.
135	ERTGTQPLG	0.502	*T*
144	VQGLTEEQR	0.089	.
161	AQMKTFDTT	0.048	.
164	KTFDTTFSH	0.336	.
165	TFDTTFSHF	0.083	.
248	ADMSTYMFK	0.009	.
290	LRFNTVFNA	0.705	*T*
296	FNAETGTWE	0.065	.
298	AETGTWECG	0.059	.
311	CLEDTAGGF	0.280	.
372	QFAITLKSY	0.420	.
398	MAMLTELRS	0.107	.
408	NAQHTQRLL	0.713	*T*
422	HPFATPLMQ	0.370	.
432	LFGITGS--	0.013	.

Serine predictions

Position	Context	Score	
8	RPKESWNHA	0.708	*S*
22	EDTESVPGK	0.054	.
28	PGKPSVNAD	0.184	.
105	KCLESGMKK	0.620	*S*
114	EMIMSDEAV	0.596	*S*
130	KRKKSERTG	0.997	*S*
167	DTTFSHFKN	0.148	.
179	PGVLSSGCE	0.033	.
180	GVLSSGCEL	0.867	*S*
187	ELPESLQAP	0.029	.
192	LQAPSREEA	0.996	*S*
200	AAKWSQVRK	0.232	.
208	KDLCSLKVS	0.808	*S*
212	SLKVSLQLR	0.003	.
221	GEDGSVWNY	0.468	.
231	PPADSGGKE	0.890	*S*
238	KEIFSLLPH	0.027	.
247	MADMSTYMF	0.225	.
256	KGIISFAKV	0.004	.
262	AKVISYFRD	0.329	.
274	EDQISLLKG	0.177	.
305	CGRLSYCLE	0.942	*S*
347	MQAISLFSP	0.029	.
350	ISLFSPDRP	0.954	*S*
375	ITLKSYIEC	0.236	.
402	TELRSINAQ	0.039	.
434	GITGS----	0.004	.

Figure 5-1B

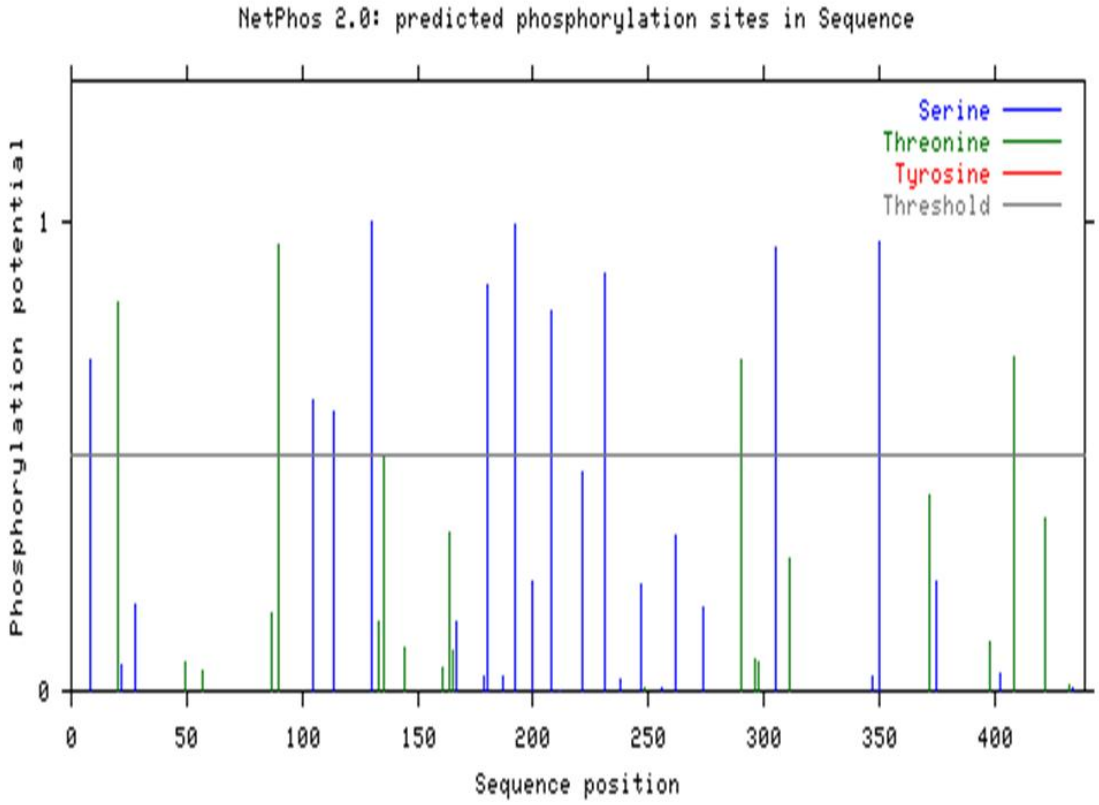
NetPhos 2.0: predicted phosphorylation sites in Sequence
Serine
Threonine
Tyrosine
Threshold
Phosphorylation potential
1
0
0
50
100
150
200
250
300
350
400
Sequence position

Figure 5-1C

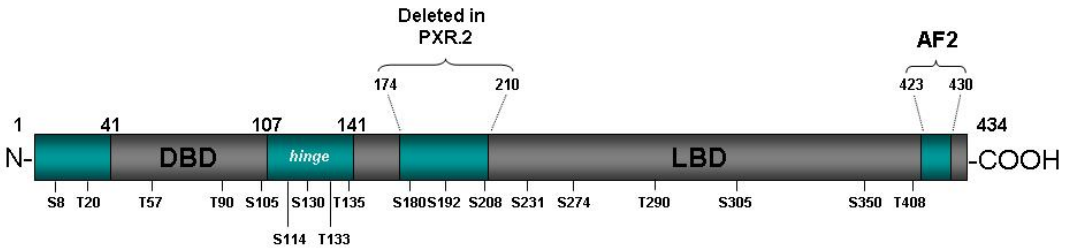

Figure 5-1. Identification of potential phosphorylation sites within the human PXR protein. Forty-eight potential phosphorylation sites within the human PXR protein were identified and scored using the NetPhos 2.0 server (A). Serine and threonine residues that scored above the 0.500 threshold were selected for site-directed mutagenesis (B). Eighteen residues located throughout the PXR protein were selected for site-directed mutagenesis (C).

Phosphomimetic mutations alter the transcriptional activity of PXR. We next sought to determine the effect that these phosphomimetic and phospho-deficient PXR mutations would have on PXR activity in cell-based reporter gene assays. Primarily, the activation of kinase signaling pathways has been shown to attenuate PXR activity. Therefore, we expected that phosphomimetic mutation at PXR phosphorylation sites would repress the activity of PXR on the XREM reporter gene. Furthermore, we expect that the phospho-deficient mutation would either increase or have no effect on PXR activity. The XREM-Luc reporter gene was used in CV-1 cells to detect changes in PXR activity based on mutations at 18 phosphorylation sites. Expression vectors encoding wild-type and mutant PXR constructs were co-transfected with XREM-Luc. Luciferase activity was monitored 48 hours post-transfection. The fold induction of each mutant compared to wild-type PXR was recorded (Table 5-2). Phosphomimetic mutations at 4 sites including S8, T57, S305 and S350 displayed attenuated PXR activity with $p<0.001$. Phospho-deficient mutations at the same five sites displayed no significant change in PXR activity. At S208, the phosphomimetic mutation attenuated PXR activity with $p<0.001$ while the phospho-deficient mutation increased PXR activity with $p<0.001$. In addition, T408 is noteworthy due to the extent to which the phosphomimetic mutation attenuated PXR activity. T90 has a high phosphorylation score of 0.949 and like T57, is located within the conserved zinc finger motifs of the PXR DBD. These 7 sites were selected for further analysis for potential phospho-specific regulation of PXR activity. The modulation of PXR activity by mutations that mimic phosphorylation suggest that

phosphorylation at those specific sites could confer a measurable functional impact by negatively regulating PXR activity. Multiple mechanisms could contribute to the impaired transactivation function these mutants including protein expression and stability, cofactor interactions, DNA binding, heterodimerization with RXRα and sub-cellular localization. Therefore we sought to elucidate the responsible mechanisms.

Table 5-2. Phosphomimetic mutations within the hPXR protein alter the transactivation capacity of hPXR in reporter gene assay. Changes in hPXR activity are represented as fold induction of hPXR mutants compared to WT on XREM-Luc.

Site	D	A
S8	**0.31****	1.17
T20	0.67	.89
T57	**0.16****	0.93
T90	0.81	0.75
S105	0.40*	0.76
S114	0.70*	1.07
S130	1.55	1.27
T133	0.78	**0.19****
T135	0.68	0.75
S180	0.79	0.56*
S192	**0.41****	**0.34****
S208	**0.44****	**2.67****
S230	0.48*	0.81
S274	0.46*	0.70*
T290	**0.23****	0.35*
S305	**0.07****	0.83
S350	**0.22****	1.52
T408	**0.06****	**0.23****

* $p<0.01$; ** $p<0.001$

Phosphomimetic mutations at T57 and T408 attenuate the ligand-inducible transactivation capacity of PXR. To determine the effect that phosphomimetic mutations at the 7 selected sites of interest has on the inducible transactivation capacity of PXR, CV-1 cells were transiently transfected with the XREM-Luc reporter gene construct and expression vectors encoding wild-type or mutant PXR. Twenty-four hours post-transfection, cells were treated with either vehicle or 10 μM rifampicin, a prototypical human PXR ligand. Luciferase activity was observed an additional 24 hours after drug treatment. As expected, wild-type PXR significantly enhanced the reporter gene activity in the presence of rifampicin. In contrast a phosphomimetic mutation at T57 abolishes the ligand-inducible activation of the reporter gene. However, the phospho-deficient mutation at T57 had no effect on the ligand-inducible activation of the reporter gene. A phosphomimetic mutation at T408 significantly attenuated the ligand-inducible activation of the reporter gene when compared to wild-type PXR. However, the phospho-deficient mutation at T408 again had no effect on the ligand-inducible activation of the reporter gene. Mutations at the additional sites of interest, S8, T90, S208, S305,and S350, did not effect the rifampicin-inducible transactivation capacity of PXR compared to wild-type (Figure 5-2).

Figure 5-2

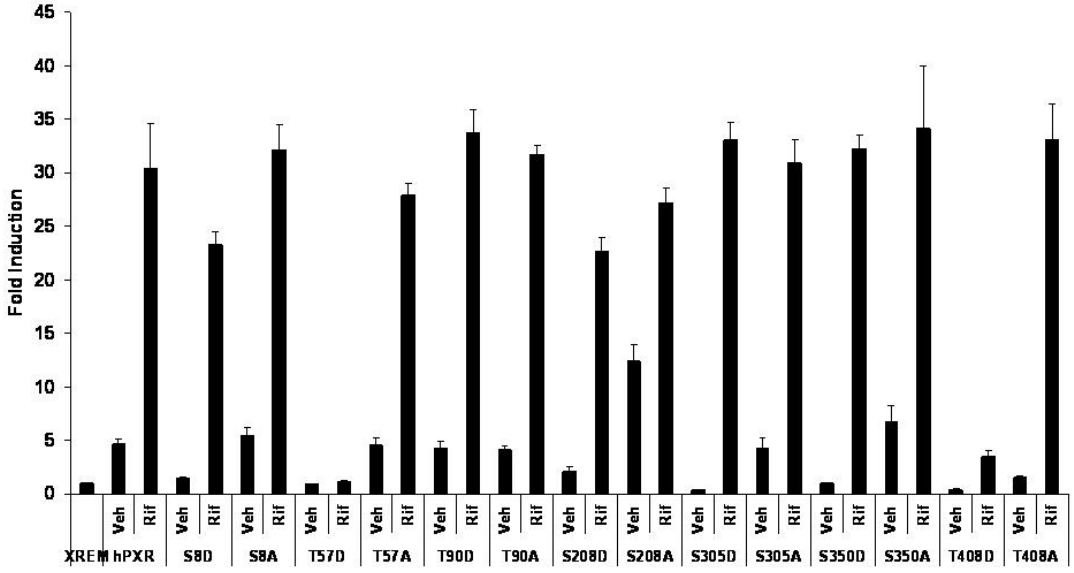

Figure 5-2. Phosphomimetic mutations at T57 and T408 attenuate the ligand-inducible transactivation capacity of hPXR. CV-1 cells were transfected with the XREM-Luc reporter gene construct and expression vectors encoding wild-type or mutant PXR. Twenty-four hours post-transfection, cells were treated with either vehicle or 10 μM rifampicin. Luciferase activity was observed an additional 24 hours after drug treatment. PXR proteins containing phosphomimetic mutations at T57 and T408 were not activated by rifampicin treatment. The data are normalized to β-galactosidase activity and represented as fold induction ± standard deviation (n=4).

Phosphomimetic mutation at T57 impairs the ability of PXR to bind to its DNA response elements. PXR-mediated transactivation of its target genes requires direct binding of the PXR protein to response elements on its target gene promoters. The PXR DBD contains two zinc finger motifs that are essential for DNA binding. T57 and T90 are located within the first and second zinc finger motifs respectively (Figure 5-3A). Phosphorylation of these residues may alter the DNA-binding capacity of PXR. Therefore, we hypothesize that the abolishment of the inducible transactivation capacity of the T57D mutant may be due to its lack of ability to bind to PXR response elements. In fact, gel shift assay results demonstrate that while both wild-type and T57A PXR bound to PXR response elements, T57D PXR failed to do so. T90D and T90A PXR were both able to bind to PXR response elements but to a lesser extent than wild-type PXR.

In vitro transcribed and translated wild-type and mutant PXR proteins were bound to a radio-labeled oligo corresponding to the ER6 PXR response element in the *CYP3A4* promoter and run on a gel. In order to verify the specificity of PXR binding to its response element, we demonstrated that the addition of increasing molar concentrations of an unlabeled wild-type, but not mutant, ER6 oligo competed with the binding of the labeled oligo. Furthermore, incubation with an anti-hPXR antibody super shifted the PXR-oligo complex. Wild-type and T57A PXR bound to the labeled oligo, whereas T57D PXR did not. T90D and T90A PXR bound to the labeled oligo, although to a lesser extent than wild-type PXR. This suggests that conservation of the T90 residue is important for DNA binding. However,

phosphorylation at T90 likely would not impact DNA binding and the functional significance of this observation is difficult to interpret. Phosphomimetic and phospho-deficient mutations at the other sites of interest, including S8, S208, S305 and S350, had no effect on oligo binding compared to wild-type (Figure 5-3B). Taken together, these observations demonstrate that phosphorylation at T57 within the PXR protein may inhibit the ability of PXR to bind to its promoter elements. Thus suggesting a mechanism whereby PXR phosphorylation could result in the impaired function of PXR.

Figure 5-3A

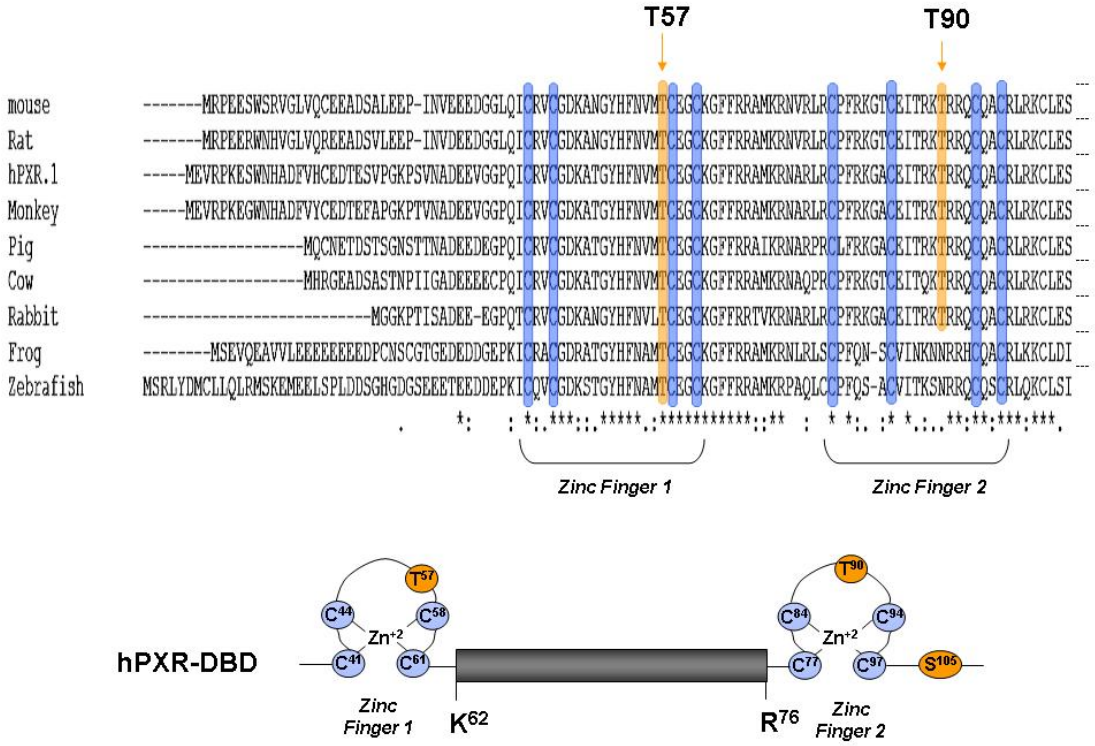
T57
T90
mouse
Rat
hPXR.1
Monkey
Pig
Cow
Rabbit
Frog
Zebrafish
Zinc Finger 1
Zinc Finger 2
hPXR-DBD
T57
C44
C58
Zn+2
C41
C61
T90
C84
C94
Zn+2
C77
C97
S105
Zinc Finger 1
K62
R76
Zinc Finger 2

Figure 5-3B

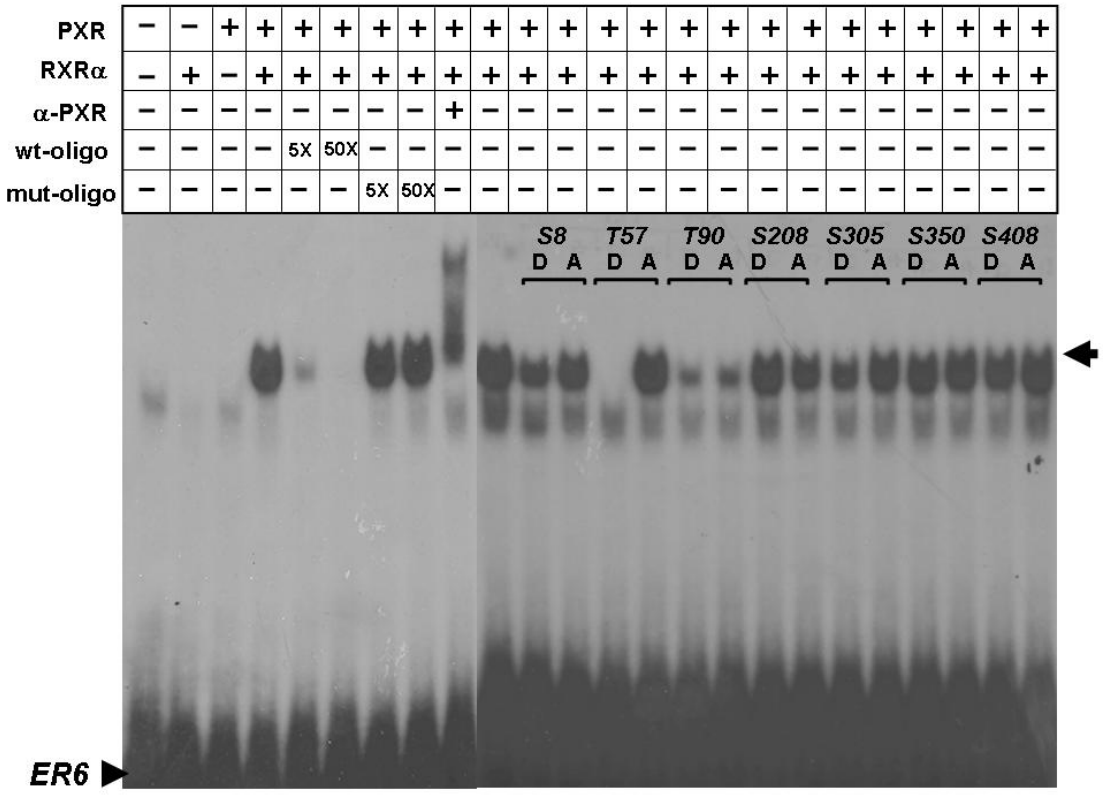

Figure 5-3. Phosphomimetic mutation at T57 abolishes the ability of hPXR to bind to its DNA response element. T57 and T90 are located within conserved zinc finger motifs within the DBD of the PXR protein (A). *In vitro* transcribed and translated wild-type and mutant PXR proteins were bound to a radio-labeled oligo corresponding to the ER6 PXR response element in the *CYP3A4* promoter and run on a gel. Assay specificity was confirmed in that the addition of a cold ER6 oligo competed for hPXR binding, while the addition of a mutant ER6 oligo did not. Addition of an anti-hPXR antibody super shifted the protein-oligo complex. Wild-type and T57A hPXR bound to the ER6 response element, whereas, T57D hPXR did not (B).

Phosphomimetic mutations at S305, S350, and T408 impair the ability of PXR to heterodimerize with RXRα. While some NRs function as monomers, most NRs are active as dimers; either as homodimers, or as heterodimers with retinoid x receptor (RXR). PXR-mediated transactivation of its target genes requires heterodimerization with RXRα. Upon ligand binding the PXR-RXR heterodimer binds to multiple sites on the PXR target gene promoters and activates gene expression. Since PXR and RXRα form a single type of heterodimeric complex, the regions that connect proteins must allow considerable flexibility to account for variations in response elements. Dimerization surfaces are located within the LBD of both PXR and RXRα. Therefore, we hypothesize that phosphomimetic mutations contained within the PXR LBD may interfere with PXR-RXR heterodimerization. Co-immunoprecipitation studies show that phosphomimetic mutations at S305, S350, and T408, contained within the LBD, do in fact, disturb PXR-RXR heterodimerization.

Expression constructs encoding flag-tagged RXRα, wild-type, and mutant PXR were *in vitro* transcribed and translated. The expression of the wild-type and mutant PXR proteins was roughly equivalent as demonstrated by western blot using an anti-hPXR antibody (Figure 5-4: top panel). The wild-type and mutant PXR proteins were co-immunoprecipitated with flag-tagged RXRα using an anti-flag antibody. The immunoprecipitated complexes were washed three times, and the presence of PXR was detected, indicating heterodimerization, using an anti-hPXR antibody. As expected, wild-type PXR heterodimerized and was co-

immunoprecipitated with RXRα using an anti-flag antibody. In order to verify the specificity of the co-immunoprecipitation in detecting PXR-RXR heterodimerization, we immunoprecipitated lysates containing, RXRα alone, PXR alone, or un-programmed cell lysate using the anti-flag antibody. We also immunoprecipitated lysate containing both flag-RXRα and wild-type PXR using a control antibody. As expected PXR was not co-immunoprecipitated or detected by western blot in any of the control reactions. In this study, heterodimerization and co-immunoprecipitation of PXR proteins containing phosphomimetic mutations within the LBD at S305, S350, and T408 was disturbed as evidenced by the decreased detection via western blot. Phospho-deficient mutation at the same sites did not appear to affect heterodimerization. Phosphomimetic mutation at T57 and T90 (located in the DBD) did not affect PXR-RXR heterodimerization, whereas mutation at S8 (located in the N-terminal region) and at S208 (located in the N-terminal region of the LBD) appear to slightly decrease PXR heterodimerization (Figure 5-4: bottom panel). These data indicate that potential phosphorylation at S305, S350, and T408 within the PXR protein may decrease PXR-RXR heterodimerization and contribute to decreased PXR activity. It is interesting to note that while S305D, S350D and T408D mutant PXR proteins did display decreased basal PXR activity, perhaps due in part to decreased PXR-RXR heterodimerization, these mutants were still functional and able to induce the expression of reporter gene activity in response to rifampicin.

Figure 5-4

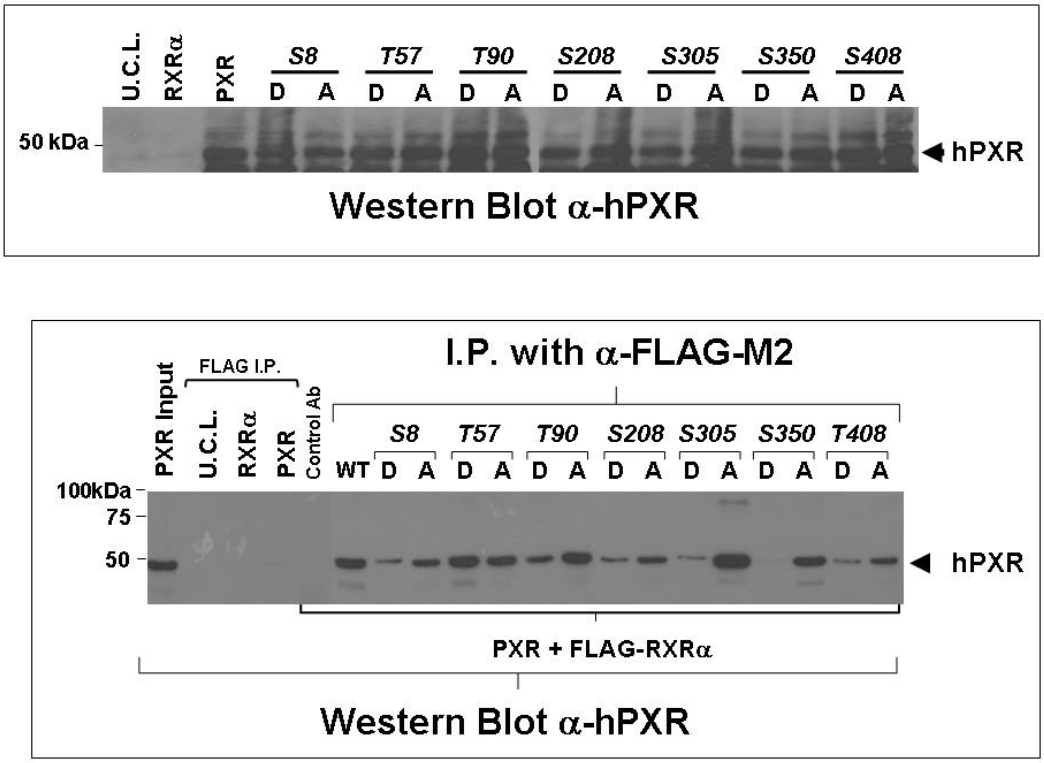

Figure 5-4. Phosphomimetic mutations at S305, S350, and T408 impair the ability of PXR to heterodimerize with RXRα. Flag-tagged RXRα, wild-type, and mutant PXR were *in vitro* transcribed and translated. The expression of the wild-type and mutant PXR proteins was analyzed by western blot using an anti-hPXR antibody (top panel). The PXR proteins were co-immunoprecipitated with flag-tagged RXRα using an anti-flag antibody. The presence of PXR was detected using an anti-hPXR antibody. Wild-type PXR co-immunoprecipitated with RXRα using an anti-flag, but not a control antibody. PXR was not detected in the immunoprecipitation of lysates containing RXRα alone, PXR alone, or un-programmed cell lysate. PXR proteins containing phosphomimetic mutations at S305, S350, and T408 were not immunoprecipitated with RXRα whereas phospho-deficient mutations at the same sites did not appear to affect heterodimerization.

Phosphomimetic and phosphor-deficient mutations at S208 and S305 differentially modulate PXR-cofactor interactions. The complete function of many NRs, including PXR, is dependent on their ability to interact with protein cofactors. For example, in the absence of ligand, PXR is associated with co-repressors such as NCoR. However, ligand-binding disrupts the PXR-co-repressor association and induces the association of PXR with co-activators such as SRC-1 and SRC-2 [5]. In addition to ligand-binding, phosphorylation is known to regulate the ability of NRs to bind to cofactors. Given that PXR interacts with transcriptional cofactors at the LBD, we hypothesize that phosphomimetic mutations contained within the PXR LBD may interfere PXR-cofactor interactions. Therefore, we used the mammalian 2-hybrid system to determine whether the loss of PXR function observed in the phosphomimetic mutations contained within the LBD could be attributed to alterations in cofactor interactions.

CV-1 cells were transiently transfected with the pFR-Luc reporter gene construct and expression vectors encoding Gal4-co-factor fusion proteins and VP16-wild-type or -mutant PXR fusion proteins. Twenty-four hours post-transfection, cells were treated with either vehicle or 10 μM rifampicin. Luciferase activity was observed an additional 24 hours after drug treatment. A phosphomimetic mutation at S208 increased the basal association of the co-repressor NCoR with PXR, whereas the phospho-deficient mutation conferred the opposite effect (Figure 5-5A). On the other hand, phosphomimetic mutation at S208 decreased the basal association of the co-activator SRC-2 with PXR and the phosphodeficient mutation increased the

association (Figure 5-5B). This differential modulation of cofactor binding to S208D and S208A hPXR is consistent with the modulation of PXR activity in the reporter gene assay. Alterations in the phosphorylation status of PXR at S208 may contribute to the modulation of PXR activity by disrupting receptor-co-factor interactions. Furthermore, phosphmimetic mutation at S305 had a similar effect of PXR-co-factor binding in that it increased the association of NCoR and decreased the association of SRC-2. Phosphomimetic mutations at S350 and T408 resulted in a general decrease in co-factor binding (Figures 5-5A and 5-5B). Ligand dependent associations of either NCoR or SRC-2 with hPXR were not disrupted by any of the selected mutations (data not shown).

Figure 5-5A

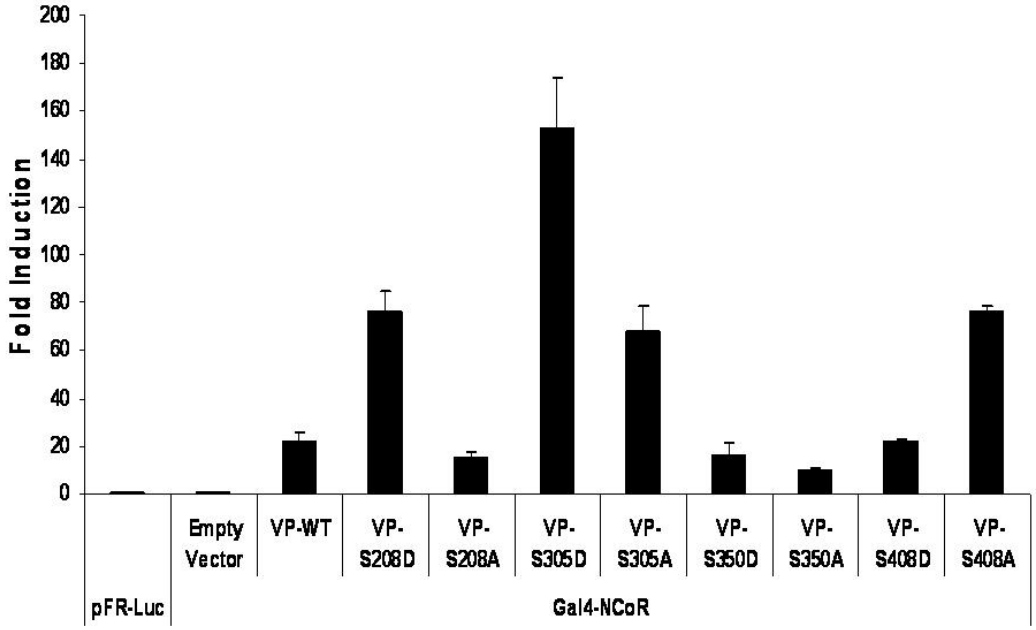
Fold Induction
200
180
160
140
120
100
80
60
40
20
0
pFR-Luc
Empty Vector
VP-WT
VP-S208D
VP-S208A
VP-S305D
VP-S305A
VP-S350D
VP-S350A
VP-S408D
VP-S408A
Gal4-NCoR

Figure 5-5B

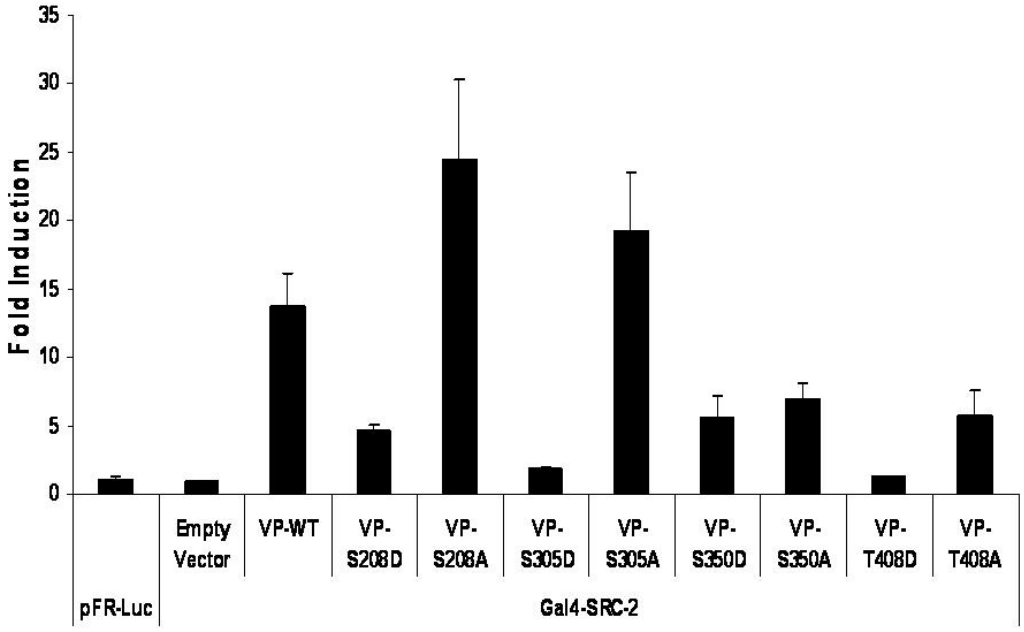

Figure 5-5. Phosphomimetic mutations at S208 and S305 alter the ability of hPXR to interact with protein cofactors. CV-1 cells were transfected with the pFR-Luc reporter gene construct and expression vectors encoding Gal4-co-factor fusion proteins and VP16-wild-type or -mutant PXR fusion proteins. Twenty-four hours post-transfection, cells were treated with either vehicle or 10 μM rifampicin. Luciferase activity was observed an additional 24 hours after drug treatment. Phosphomimetic mutations at S208 and S305 increase the strength of interaction between PXR and the co-repressor NCoR (A) and decrease the strength of interaction between PXR and the co-activator SRC-2 (B). The data are normalized to β-galactosidase activity and represented as fold induction ± standard deviation (n=4).

5.4 Discussion

Although the primary mode of regulation of NRs is ligand-binding, increasing amounts of evidence show that cell signaling and the modulation of NR and co-factor phosphorylation statuses are critical in determining the NR response to changes in environmental stimuli [3, 4]. Phosphorylation has been implicated in the modulation of multiple aspects of NR activity including transcriptional activity, protein expression and stability, sub-cellular localization, heterodimerization, DNA binding, and cofactor interactions.

It is well documented that changes in environmental conditions such as inflammation, diabetes, obesity, malnutrition, and alcohol consumption all result in the modulation of the expression and activity of drug metabolizing enzymes [24, 25]. More specifically, the expression and activity of CYP3A4, a hepatic enzyme responsible for the oxidative metabolism of roughly 60% of all prescription drugs, is rapidly repressed in response to inflammation [26]. PXR is a master regulator of the drug-inducible transcription of not only the *CYP3A4* gene, but also of genes that encode additional drug metabolizing enzymes and drug transporter proteins. Given the potential for drug-environment or drug-disease interactions, it is important to understand the likely role of PXR in mediating transcriptional repression in response to environmental stimuli and pathological conditions. While much is known about the PXR ligand-inducible regulation of target genes, relatively little is known regarding the regulation of PXR activity by signaling pathways and post-translational modifications. We and others are interested in understanding the molecular

mechanisms by which signal transduction pathways interface with PXR to mediate the repression of PXR target genes. Understanding these mechanisms that regulate the drug metabolism program is critical for the development of effective clinical therapeutic strategies and to avoid potentially dangerous drug interactions.

It is known that PKA, PKC, and CDK2 are involved in the regulation of PXR activity [17-20]. In primary cultures of mouse hepatocytes, treatment with phorbol ester, a PKC activator, dramatically represses the expression of cyp3a11 [19]. However, treatment of mouse hepatocytes with 8Br-cAMP, a PKA activator, potentiates the expression of cyp3a11 [17]. Cell-based mammalian 2-hybrid experiments suggest that the modulation of cyp3a11 expression by PKC and PKA is due in part, to the increased association of PXR with the cofactors NCoR and SRC-1 respectively [17, 19]. However, it remains unclear as to whether the activation of PKA or PKC signaling modulates PXR activity via direct phosphorylation of PXR, direct phosphorylation of a protein cofactor, or an entirely different mechanism. Interestingly, while the activation of PKA signaling potentiates the expression of cyp3a11 in mouse hepatocytes, it represses the expression of CYP3A4 in human hepatocytes [18]. Treatment of hepatocytes isolated from a liver specific 'humanized' PXR transgenic mouse model with 8Br-cAMP, resulted in the potentiation of cyp3a11 expression. However, it is noteworthy that the activation of PKA signaling potentiated the expression of cyp3a11 in mouse hepatocytes in a synergistic manner, whereas the effect in 'humanized' PXR hepatocytes appeared to be additive [18]. This evidence suggests that the species-specific interface between

PXR activity and PKA signaling is not entirely contained within the PXR protein. Nonetheless, it is known that human PXR exists as a phospho-protein *in vivo* and is a good substrate for PKA *in vitro* [18]. Therefore, we can speculate that the modulation of PXR activity by PKA or other kinase signaling pathways is due to a combination of factors that may include the phosphorylation of PXR or a PXR-interacting protein. Another recent study has shown that the activation of CDK2 leads to the attenuation of hPXR activity in cell-based reporter gene studies [20]. The same study indicates that CDK2 can phosphorylate human PXR *in vitro* at more the one site. Furthermore mutagenesis analysis identified S350 as a potential site for CDK2 phosphorylation [20].

It is clear that the activation of signaling pathways modulates PXR activity; however the extent to which the phosphorylation of PXR is involved is unknown. A recent study sought to characterize the effect of a phosphomimetic mutation at T57 on the activity of human PXR [21]. T57 is highly conserved throughout the NR super-family in the first zinc finger motif of the DBD. The T57 phosphomimetic mutant of hPXR loses its transactivation function and displays a punctate nuclear distribution. Gel shift assays suggest that this may be due to the impaired ability of the mutant PXR to bind to its DNA response elements [21]. The same study identified T57 as a potential phosphorylation site for p70 S6K and showed that p70 S6K can phosphorylate hPXR *in vitro* [21]. Given the likelihood that PXR is regulated as a phospho-protein, and given the information that T57 and S350 have been identified as

potential phosphorylation sites within the PXR protein, we sought to systematically identify and characterize potential phosphorylation sites within the PXR protein.

While ligand binding is the primary mechanism of PXR activation, it is likely that phosphorylation is involved in fine-tuning PXR activity in response to environmental stimuli. In the current study, we identified 18 likely PXR phosphorylation sites using *in silico* consensus site prediction methods. Of the 18 sites, 7 were chosen for further characterization. Phosphomimetic mutations at each of the 7 sites resulted in the repression of PXR activity ($p<0.001$), whereas, the phospho-deficient mutations had either no effect or increased PXR activity. There are multiple mechanisms by which phosphorylation at a specific site could result in decreased PXR activity including the impairment of transcriptional activity, DNA binding, heterodimerization, cofactor interactions, or sub-cellular localization. We show that phosphomimetic mutations at T57 and T408 impair the ligand-inducible transactivation capacity of PXR whereas phospho-deficient mutations at those sites have no effect on PXR transactivation. Similar to previous reports, we observed that a phosphomimetic mutation at S350 resulted in the decreased basal expression of PXR in cell based reporter gene assays; however, we did not observe an attenuation of ligand-induced PXR activity as reported by Lin *et al.* [20]. Our data also suggest that the lack of PXR activity displayed by the T57D PXR mutant is due to its inability to bind to PXR response elements. This result is consistent with the previous report characterizing a phosphomimetic mutation at T57 [21]. In addition, given that T57 and T90 are highly conserved residues located within the zinc fingers of the PXR

DBD, it is not astounding to speculate that phosphorylation at those sites would disrupt DNA binding. However, both T90D and T90A mutants retained their ability to bind to DNA, albeit to a lesser extent than wild-type PXR. While T90 appears to be a critical residue for DNA binding, our data indicate that phophorylation at this site would may not have an impact on PXR DNA binding and activity. Furthermore, phosphomimetic mutations at S305, S350, and T408 inhibit PXR-RXR heterodimerization, thus providing a mechanism by which phosphorylation at those sites could result in decreased PXR activity. Mammalian 2-hybrid experiments suggest that phosphorylation of PXR at S208 or S305 could result differential modulation of PXR-co-factor interactions and in subsequent PXR activity.

Taken together, our data provide a systematic identification and characterization of potential phosphorylation sites within the PXR protein. We show that potential phosphorylation at sites throughout the PXR protein could modulate PXR activity by altering either one or a combination of the following parameters: transactivation capacity, DNA binding, heterodimerization, or cofactor interactions. However, there are some inconsistencies present in this data set. For example, a phosphomimetic mutation at S350 inhibits the ability of PXR to heterodimerize with RXRα in co-immunoprecipitation assays but does not affect its ability to bind to its response element with RXRα in gel shift assays. In addition, both phosphomimetic and phospho-deficient mutations at T90 slightly impair the ability of PXR to bind to its response element in gel shift assays but do not effect PXR activity in reporter gene assays. Thus, it is difficult to interpret the extent to which the alterations in the

parameters of PXR activity, as measured in this study, are functionally significant. Furthermore, it is unknown as to whether or not phosphorylation at any of the characterized sites is physiologically significant. *In silico* analysis reveals that many of the conserved PXR phosphorylation sites are potentially good substrates for an array of kinases (Table 5-3). However, preliminary experiments performed in our lab, utilizing constitutively active kinase expression vectors in cell-based reporter gene assays, have been unsuccessful in identifying sites that are responsive to specific kinases (data not shown). Further studies are required to determine the physiological connection between the activation of kinase signaling pathways and altered PXR activity as well as to determine the extent to which the direct phosphorylation of PXR is involved.

Table 5-3. ***In silico*** **identification of conserved hPXR phosphorylation sites that are potentially good substrates for specific kinases.**

Phosphorylation Site	*Kinase Prediction*
S8	*PKA* *PKG*
T57	*CK1* *p70 S6K*
T90	*PKA* *PKC*
S208	*PKC*
S305	*CK2* *MAPK* *PKA*
S350	*CDK2* *CDK5* *CK1* *MAPK*
T408	*PKC*

The full activity of the PXR signaling pathway is dependent on both crosstalk with other signaling pathways and on PXR-cofactor interactions. For example, it is known that PXR activation suppresses the hepatic immunological response. On the other hand, inflammation is known to decrease PXR-mediated gene activation. The evidence suggests that the activation of the NF-κB signaling pathway during inflammation interferes with PXR heterodimerization [14]. Furthermore, the activation of PXR has recently been shown to decrease energy metabolism and increase hepatic triglyceride levels. The crosstalk between metabolic signaling pathways and PXR is thought to be due to direct interactions between PXR and the transcriptional regulators CREB, PGC-1α and FOXO [8, 27-29]. The molecular basis for the crosstalk between PXR and these metabolic pathways is unknown, although kinase signaling events are likely involved. It is also well known that the activation of kinase signaling resulting in the phosphorylation of protein cofactors, such as SRCs, PGCs, and NCoR, disrupts NR-cofactor interactions. Since NRs share many of the same cofactors, it is likely that the direct phosphorylation of cofactors could contribute to altered PXR activity in response to kinase activation. Altogether, the interface between signal transduction pathways and PXR activity is complex and the physiological relevance of PXR phosphorylation is undefined. It is most likely that the phosphorylation of PXR interacting proteins in addition to the potential phosphorylation of PXR itself contributes to alterations in PXR activity. Even so, understanding the mechanisms by which environmental stimuli and signal

transduction pathways modulate the expression of PXR target genes is critical for the development of safe and effective therapeutic strategies.

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Chapter 6: The Future Outlook for PXR

6.1 The Significance of PXR

Normal homeostasis requires the detoxification and elimination of xenobiotics from the body through the combined action of the phase I oxidative cytochrome-P450 (CYP) enzymes, the phase II conjugating enzymes, and the membrane transporter proteins in liver. It is a long-standing observation that the expression of the genes encoding many drug-metabolizing enzymes (DMEs) can be dramatically induced by exposure to certain xenobiotic compounds [1]. Shortly after its discovery, PXR was classified as a receptor that is activated by a wide variety of xenobiotic compounds to activate the expression of genes involved in biotransformation, at long last providing the molecular basis for the induction of DME gene expression by xenobiotics [2, 3]. Therefore, PXR activation is a principle defense mechanism protecting the body from toxic assault. However, the discovery that the activation of PXR and the subsequent induction of DMEs can result in the accelerated metabolism of other medications, demonstrates that PXR activation also represents the basis for an important class of drug-drug interactions [3, 4].

This phenomenon is a serious concern for patients taking multiple medications with small therapeutic indices as alterations in drug metabolic rates in patients can often have life-threatening consequences. Therefore, in order to develop safe and effective therapeutic strategies, it is critical to systematically screen new drug candidates and clinically used drugs for their ability to activate PXR. Ideally, drug candidates would not activate PXR, and those that do can be replaced with

compounds that have similar therapeutic efficacy, but lack the ability to activate PXR. In addition, evidence presented in this dissertation and elsewhere shows that herbal compounds such as St. John's Wort, Tian Xian, guggulsterone, and others can modulate the activity of PXR and may contribute to an herb-drug interaction [5-8]. Herbal products are readily available over-the-counter and are not regulated for biological activity and side effects to the extent that prescription drugs are. Therefore, it is important to continue to screen natural products for PXR activity and to educate the general public on this issue.

While the primary function ascribed to PXR is the homeostatic control of steroids, bile acids, and xenobiotics, more recent research indicates a suppressive role for activated PXR in both gluconeogenesis and inflammation that is mediated through crosstalk with the forkhead transcription factor FOXO1 and the inflammatory mediator NFκB, respectively [9, 10]. Additional research indicates a key role for PXR in the development of hepatic steatosis and in the homeostasis of vitamin D [11-13]. Therefore, in addition to drug-drug interactions, PXR activation may also represent the basis for drug-induced pathological conditions such as hypoglycemia, impaired immune function, hepatic steatosis, or osteomalacia. Further research is required to determine the extent to which PXR activation contributes to the development of such conditions.

6.2 PXR and Kinase Signaling

Although the primary mode of regulation of NRs is ligand-binding, increasing amounts of evidence show that cell signaling and the modulation of NR and co-factor phosphorylation statuses are critical in determining the NR response to changes in environmental stimuli [14, 15]. It is well documented that changes in environmental conditions such as inflammation, diabetes, obesity, malnutrition, and alcohol consumption all result in the modulation of the expression and activity of DMEs (Figure 6-1) [16, 17]. Understanding the mechanisms that regulate the expression of drug metabolizing enzymes is critical in the development of effective clinical therapeutic strategies and to avoid potentially dangerous drug interactions.

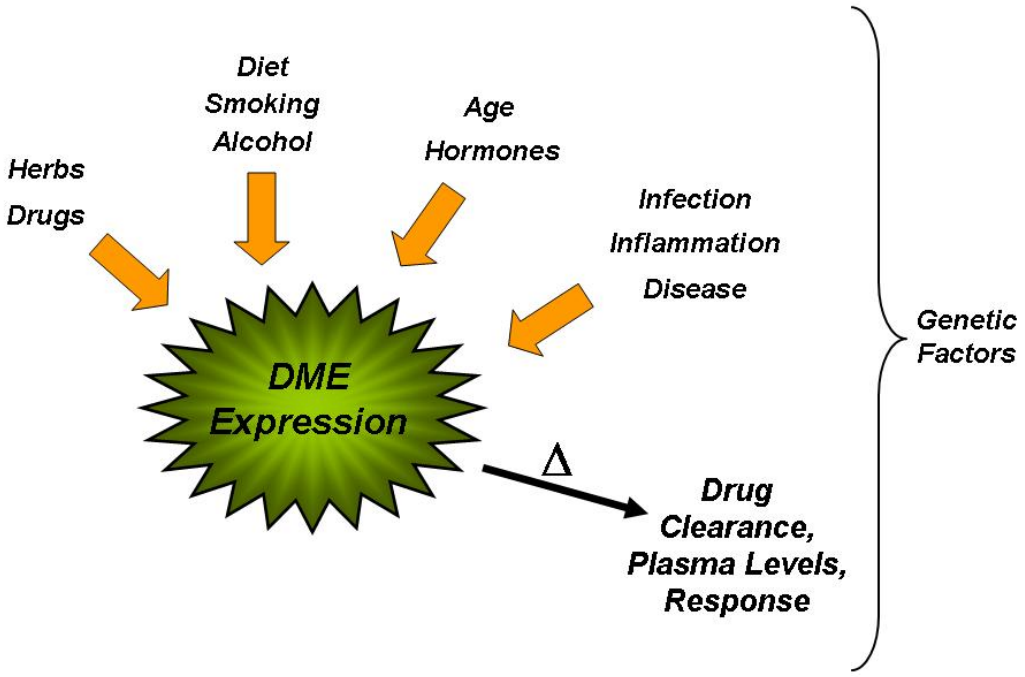

Figure 6-1. Environmental stimuli modulate the expression of drug-matabolizing enzymes. Changes in environmental conditions including exposure to xenobiotics, lifestyle choices, age, disease, and pathological conditions all result in the modulation of the expression and activity of DMEs. The mechanism by which signaling pathways interface with PXR and affect its overall responsiveness to environmental stimuli is an exiting area of future research.

Drug-inducible DME gene expression is known to be responsive to kinase signaling pathways; however, the exact mechanism by which these pathways intersect with PXR is unknown. The activation of PKA, PKC, CDK2, and p70 S6K signaling pathways result in the attenuation of PXR activity and PXR is a good substrate for these kinases *in vitro* [18-21]. The results of these studies suggest that the activity of PXR is modulated by changes in phosphorylation within the cell, although direct phosphorylation of PXR was not demonstrated *in vivo*. Data presented in this dissertation provides the first evidence that PXR exists as a phospho-protein *in vivo* and that its phosphorylation status is modulated in response to the activation of a kinase signaling pathway [22]. Furthermore, given that PXR activity is noticeably regulated by the activation of kinase signaling pathways, we and others have sought to understand the mechanism by which phosphorylation dependent events modulate PXR signaling. Data presented in this dissertation systematically show that phosphorylation at consensus sites throughout the PXR protein could result in the modulation of multiple aspects of PXR activity including transcriptional activity, heterodimerization, DNA binding, and cofactor interactions. This suggests that PXR could be involved in integrating external signals via phosphorylation. In addition, the post-translational modification of co-factors and NR-interacting proteins, such as RXRα, are critical in modulating the activity of many NRs in response to signaling pathways [14, 15]. Future studies are required to determine the impact that the direct phosphorylation of PXR or PXR-interacting proteins may have on PXR transactivation capacity.

It is noteworthy that, similar to the PXR ligand response, a species-specific effect for the modulation of PXR activity by the PKA signaling pathway is reported in this dissertation. We show that while PKA activation potentiates the drug-inducible expression of *Cyp3a11* in mouse hepatocytes, treatment of hepatocytes with 8-Br-cAMP serves as a repressive signal in both human and rat hepatocytes [22]. Pharmaceutical companies commonly screen for PXR activation by drug candidates in both rodent and human species in order to avoid future drug-drug interactions. Future studies that contribute to understanding the mechanism by which signaling pathways interface with PXR across species will be useful in the development of more accurate activation assays in order to predict and prevent potentially lethal drug-drug interactions.

6.3 PXR as a Drug Target

As mentioned in chapter 2, in addition to drug metabolism, PXR has been implicated in the regulation of bile acid and bilirubin homeostasis, glucose and lipid homeostasis, the inflammatory response and in cancer. Therefore, compounds that target PXR may be useful in the treatment of diseases that result from the disturbance of such homeostatic pathways. In fact, there are multiple clinical examples in which PXR ligands have been used in the treatment of disease. For example, rifampicin has been used for the treatment of jaundice and pruritus associated with cholestatis [23, 24]. Budesonide, an anti-inflammatory drug used in the treatment of inflammatory bowel disease, has been recently identified as a PXR ligand [25]. In addition, rifaximin, which was initially approved for the treatment of travelers' diarrhea, has

been used off-label for the treatment of inflammatory bowel disease and was identified as a gut-specific PXR activator [26]. Further studies are required to assess the potential role of PXR activation in such therapeutics.

Rifampicin treatment has also been known to induce side-effects such as hepatic steatosis [27]. Given emerging evidence for the role of PXR in lipid homeostasis and hepatic steatosis, the inhibition of PXR may represent a novel steatosis treatment strategy [12, 13]. Finally, recent evidence shows that PXR is up-regulated and appears to promote tumor growth in certain human cancers [28-31]. Again, future studies are required to assess the potential role of PXR inhibition in the treatment of diseases such as steatosis and cancer.

The ability to modulate the activity of PXR using small lipophilic ligands makes it an attractive drug target. However, PXR regulates the expression of multiple target genes that are involved in several physiological processes. One of the challenges in targeting PXR is separating the desired therapeutic effects from the undesirable side effects. For example, the unwanted activation of PXR represents the basis for drug-drug interactions and in order for PXR to be an effective therapeutic target; the activation of a potential therapeutic-target gene must be separated from the activation of genes involved in drug metabolism. One promising strategy in the development of drugs that target PXR is to take advantage of selective receptor modulators (SRMs) that exhibit agonistic or antagonistic activity in a cell- or tissue-dependent manner. The expression profile of co-regulator proteins and signaling pathways within different cell types likely contributes to the differential activities of

SRMs. Another strategy is to attempt to target PXR in a promoter-specific manner. It is known that steroidal compounds preferentially induce PXR activity towards the *CYP3A* promoter, whereas anti-cancer agents preferentially induced the *MDR1* promoter. The mechanism for this differential promoter activation was traced to the differential recruitment of co-activator proteins [32]. In this manner, an ideal drug candidate would activate PXR on the promoters of target genes involved in the treatment of disease, but would not activate PXR on the promoters of genes involved in drug metabolism.

As our understanding of PXR signaling increases, so does our appreciation of the complexity of its regulation. It is likely that the clinical therapeutics will include strategies that not only target PXR, but also co-regulator proteins and signaling pathways that are critical in the modulation of its function. Future research that contributes to a better understanding of the co-regulator proteins and signaling pathways that interface with PXR may provide alternative drug therapies toward that end.

6.4 Concluding Remarks

In the past ten years, PXR has moved from an orphan receptor to a well-characterized xenobiotic sensor and a putative drug target. We now face new challenges to deepen our understanding of the basic functions of PXR in human biology, as well as how the receptor might be harnessed in a clinical setting. The role that distinct ligands play in the PXR-mediated regulation of tissue-, promoter-, and co-factor-specific transcriptional events represents a new direction toward that end.

The identification of novel ligands and target-genes continues to be an important aspect of PXR research. However, the mechanism by which signaling pathways interface with PXR and affect its overall responsiveness to environmental stimuli is emerging as a key area of study for this receptor. In addition, the potential impact of sites of phosphorylation on the action and stability of PXR and PXR-interacting proteins warrants detailed attention. Finally, the search for selective PXR modulators might provide novel therapeutic tools to target this noteworthy receptor in the treatment of human diseases.

6.5 References

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