UBIQUITINATION AND SUMOYLATION OF PREGNANE X RECEPTOR

By

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Abstract

Pregnane X receptor (PXR, NR1I2) is a ligand-activated nuclear receptor (NR) superfamily member expressed at high levels in the liver and intestine of mammals. PXR can be activated by a broad range of structurally diverse xenobiotics and endobiotics. As a key regulator of xenobiotic metabolism and clearance, activated PXR up-regulates the expression of genes encoding phase I (oxidation) and phase II (conjugation) metabolizing enzymes and phase III transporters to increase the metabolism and clearance of drugs and xenobiotics from the body, thus protecting the body from potential toxic insults. Besides xenobiotic metabolism and clearance, activation of PXR also involves in the regulation of many other important biochemical pathways, like inflammation and bile acid homeostasis. While ligand-binding is the primary mechanism for NRs activation, recent research indicates that post-translational modifications of NRs also help to determine their activities under different physiological conditions and represent new modes of regulation for NRs.

Studies on post-translational modifications of PXR have just begun to emerge, how post-translational modifications regulate PXR activity is not well-understood. This dissertation focuses on ubiquitination and SUMOylation of PXR. These post-translational modifications of PXR were characterized and their effects on PXR activities were studied in both primary cultures of hepatocytes and immortalized cell lines. Data presented here indicate that PXR is a target of the ubiquitin proteasome system, and inhibition of proteasome activity decreases the transactivation of PXR. The E3s and SENPs (Sentrin-specific Protease) that regulate PXR SUMOylation and de-SUMOylation are identified. Utilizing the newly identified SENPs, SUMOylation is further confirmed to be indispensable for PXR

to repress inflammatory response.   Furthermore, the crosstalk between ubiquitination and

SUMOylation at the level of PXR is explored.   Our data indicate that SUMOylation

increases the presence of ubiquitinated PXR, and many other substrates of ubiquitin.   Taken

together, this dissertation contributes to the understanding of post-translational modifications

of PXR and their regulatory effects on drug metabolism and inflammation, which is expected

to produce new opportunities for the development of novel and safe therapeutic strategies.

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List of Abbreviations

Aβ: Amyloid β-peptide

ABCA1: ATP Binding Cassette Transporter A1

ACBP: Acyl-CoA Binding Protein

AD: Alzheimer's Disease

AF-1: Activation Function 1

AF-2: Activation Function 2

AMP: Adenosine Monophosphate

AMPK: AMP-activated Protein Kinase

APL: Acute Promyelocytic Leukemia

APP: Amyloid Precursor Protein

AR: Androgen Receptor

ATP: Adenosine Triphosphate

BRCA1: Breast Cancer 1, Early Onset

CAR: Constitutive Androstane Receptor

CDK: Cyclin-dependent Kinase

ChIP: Chromatin Immunoprecipitation

CHIP: C-terminus of Hsc70 Interacting Protein

CK: Caseine Kinase

COX2: Cyclooxygenase 2

CPT1a*:* Carnitine Palmitoyltransferase 1A

CRE: Cyclic AMP Response Element

CREB: Cyclic AMP Response Element Binding Protein

CYP: Cytochrome P450

DAX-1: Dosage-sensitive Sex Reversal, Adrenal Hypoplasia Critical Region, on Chromosome X, Gene 1

DBD: DNA-binding Domain

DSS: Dextran Sulfate Sodium

ER: Estrogen Receptor

ERK: Extracellular Signal-regulated Protein Kinase

FOXA2: Forkhead Box Transcription Factor A2

FOXO1: Forkhead Box Transcription Factor O1

FXR: Farnesoid X Receptor

G6Pase: Glucose 6 Phosphatase

GR: Glucocorticoid Receptor

GRE: Glucocorticoid Response Element

GSK3β: Glycogen Synthase Kinase 3β

GST: Glutathione S Transferase

HAT: Histone Acetyltransferase

HDAC: Histone Deacetylase

HGF: Hepatocyte Growth Factor

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

HNF4α: Hepatocyte Nuclear Factor 4 Alpha

HRE: Hormone Response Element

HSP: Heat Shock Protein

IGP: Imidazoleglycerol Phosphate

IL: Interleukin

IL-1Ra: Interleukin 1 Receptor Antagonist

JNK: C-jun N-terminal Kinase

LBD: Ligand-binding Domain

LCA: Lithocholic Acid

LEF: Lymphoid Enhancer-binding Factor

LPS: Lipopolysaccharide

LRH-1: Liver Receptor Homolog 1

LXR: Liver X Receptor

MAPK: Mitogen-activated Protein Kinase

MDM2: Mouse Double Minute 2 Homolog

MDR1: Multi-drug Resistance 1

MG132: N-Benzyloxycarbonyl- L-Leucyl-L-Leucyl-L-Leucinal

MR: Mineralocorticoid Receptor

MRP: Multi-drug Resistance Protein

NCoR: Nuclear Receptor Co-repressor

NF-κB: Nuclear Factor kappa-light-chain-enhancer of Activated B Cells

NR: Nuclear Receptor

Nurr1: Nuclear Receptor Related 1 Protein

OAT1：Organic Anion Transporter 1

OATP2: Organic Anion Transporting Polypeptide 2

OATP1A2: Organic Anion Transporting Polypeptide 1A2

OCTN1：Organic Cation Transporter 1

PCN: Pregnenolone 16α-carbonitrile

PEPCK: Phosphoenolpyruvate Carboxykinase

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

Pgp: P-glycoprotein

PIAS: Protein Inhibitors of Activated STAT

PIC: Pre-initiation Complexes

PKA: Cyclic-AMP-dependent Protein Kinase

PPAR: Peroxisome Proliferator Activated Receptor

PML: Promyelocytic Leukemia

PR: Progesterone Receptor

PTM: Post-translational Modification

PXR: Pregnane X Receptor

PXR-KO: Pregnane X Receptor Knockout

RANBP2: Ran Binding Protein 2

RAR: Retinoic Acid Receptor

RIF: Rifampicin

RNF: RING Finger Protein

ROR: RAR-related Orphan Receptor

RXR: Retinoid X Receptor

S14: Thyroid Hormone–responsive SPOT14 Homolog

SAGA: Spt-Ada-Gcn5-acetyltransferase

SCD-1: Stearoyl-CoA Desaturase-1

SDF-4: Stromal Cell Derived Factor 4

SENP: Sentrin-specific Protease

SF-1: Steroidogenic Factor 1

SHP: Small Heterodimeric Partner

SMRT: Silencing Mediator for Retinoid or Thyroid Hormone Receptors

SRC: Steroid Receptor Coactivator

STAT: Signal Transducers and Activators of Transcription

SUG1: Suppressor for Gal1

SULT: Sulfotransferase

SUMO: Small Ubiquitin-like Modifier

TAF: TBP-associated Factor

TBP: TATA-box Binding Protein

TCF-4: T-cell Factor-4

TCPOBOP: 1, 4-bis [2-(3, 5-dichloropyridyloxy)] benzene

TFIID: Transcription Factor-IID

TLR: Toll-like Receptor

TNFα: Tumor Necrosis Factor Alpha

TR: Thyroid Receptor

UGT: Uridine-5′-diphosphate Glucuronosyl Transferase

VDR: Vitamin D Receptor

VDRE: Vitamin D Response Element

XREM: Xenobiotic Responsive Enhancer Module

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Chapter1: Introduction

1. 1 Nuclear Receptors

Nuclear receptors (NRs) are a family of ligand-activated transcription factors. They can sense the presence of their ligands, like steroids, retinoids, and hormones, to regulate the expression of their target genes. Through this mechanism, NRs participate in development, metabolism, and homeostasis among other processes, and their activities are closely associated with diseases related to these processes (1). Moreover, since most ligands that bind to NRs are small lipophilic molecules that can be easily synthesized and modified, NRs have gained great attention as therapeutic targets. Currently, molecules that target NRs make up approximately 13% of U.S. Food and Drug Administration (FDA) approved drugs (2). Some examples of NRs as therapeutic targets include androgen receptor (AR, NR3C4) antagonists in the treatment of prostate cancer, estrogen receptor α (ERα, NR3A1) antagonists in the treatment of breast cancer, and peroxisome proliferator-activated receptor γ (PPARγ, NR1C3) agonists in the treatment of type II diabetes. Their fundamental roles and their potential as therapeutic targets make studies of NRs of great importance.

1.1.1 The Structure of Nuclear Receptors

A characteristic multi-domain structure is shared by most NRs. From N-terminus to C-terminus, there is N-terminal modulatory domain (A/B region), DNA binding domain (DBD, C region), hinge region (D region), ligand binding domain (LBD, E region), and C-terminal domain (F region). The N-terminal modulatory domain is the least conserved

Figure 1-1
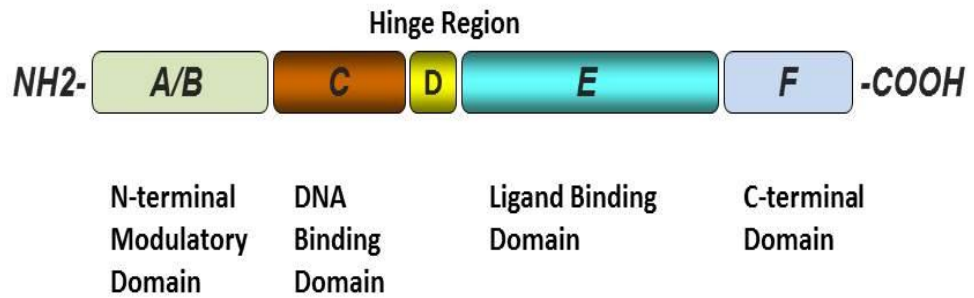
Figure 1-1. Structure of Nuclear Receptors. A characteristic multi-domain structure is shared by most NRs. From N-terminus to C-terminus, there is N-terminal modulatory domain (A/B region), DNA binding domain (DBD, C region), hinge region (D region), ligand binding domain (LBD, E region), and C-terminal domain (F region).

region among different domains within the NR family. AF-1 (Activation Functions 1) sequence resides in this domain. AF-1 functions as a ligand-independent transactivation domain. The activation capacity of AF-1 varies considerably between different NRs. The DBD binds to DNA, as indicated by its name. The structure of DBD is highly conserved within the NR family. There are two zinc fingers in DBD that can bind to specific DNA sequences and position NRs to their binding sites. The zinc finger binding sequences are disparate among different NRs and these diverse sequences are known as hormone response elements (HREs). HREs locate not only in the vicinity of target gene promoters, but also in intronic and enhancer regions. The DBD is connected with the LBD by the hinge region, which contains a nuclear localization signal. For most NRs, the LBD is structured as a three-layered antiparallel α-helical sandwich by eleven or twelve α-helices and two to four β-strands. Three parallel α-helices together with β-strands are flanked by two layers of α-helices on both sides, forming the sandwich structure. The ligand binding pocket of LBD is located below the three parallel α-helices (3). The shape of the ligand binding pocket, hydrophobic interactions, hydrogen-bonding, and the steric size together with other factors determine the binding between ligands and LBDs (4). Compared to DBD, the structure of LBD is less conserved within the family. The ligand-dependent transcription activator, AF-2, is located within the LBD. In most cases, activation of AF-2 is much stronger than AF-1. AF-1 synergizes with AF-2 to achieve full transcriptional activity (5). However, there are exceptions. For instance, AF-1 is responsible for the majority of AR activity (6). The C-terminal domain is highly variable in sequence between different NRs and the structure and function of this domain are not well-known yet. Recent studies indicate that the

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C-terminal domains are also closely related to NR activities. Deletion of the C-terminal domain of ERα eliminates the transactivation of ERα to certain agonists. The C-terminal domain of hepatocyte nuclear factor 4 alpha (HNF4α, NR2A1) interacts with the silencing mediator for retinoid or thyroid-hormone receptors (SMRT), and contributes to the regulation of HNF4α activities (7, 8).

It is generally believed that NRs are specific to metazoans (9). Based on structural similarity mentioned above, 48 NRs are identified in the human genome. The numbers of NRs in mice and rats are similar to human, with 49 NRs in mice and 47 in rats. In *Drosophila*, there are 21 receptors. However, there are up to 284 receptors in *C. elegans* (10, 11). Exceptions in NR structure do exist. Dax1 (dosage-sensitive sex reversal, adrenal hypoplasia critical region, on chromosome X, gene 1, NR0B1) and SHP (small heterodimer partner, NR0B2), two atypical orphan members of the NR subfamily, lack DBDs (12, 13). Nurr1 (Nuclear receptor related 1 protein, NR4A2) and the drosophila NR DHR38 lack detectable ligand binding pockets within their LBDs (14, 15). Many NRs are identified by sequence similarity to known receptors. At the time of their identification, cognate ligands to some newly identified NRs remain unknown, and these NRs are referred as "orphans". When ligands for orphan NRs are identified, the "orphans" will be adopted. Pregnane X receptor (PXR, NR1I2), the focus of this dissertation, is one well-known adopted orphan NR.

NRs can bind to HREs as monomers or as either homodimer or heterodimer with retinoid X receptor (RXR, NR2B). Based on their dimerization pattern and DNA binding properties, NRs can be divided into four different types. Type I NRs function as

homodimers and bind to DNA half-sites organized as inverted repeats. Within this group are

steroid receptors, including estrogen receptor α/β (ERα/β, NR3A1/2), glucocorticoid receptor

(GR, NR3C1), mineralocorticoid receptor (MR, NR3C2), progesterone receptor (PR, NR3C3),

and androgen receptor (AR, NR3C4). In the absence of ligands, type I NRs are sequestered

in cytosol by chaperone proteins like heat shock proteins (HSPs). Ligand binding can lead

to a conformation change of NRs and dissociate chaperone proteins. After the dissociation,

liganded NRs translocate to the nucleus and bind to DNA to regulate the expression of their

target genes. Type II NRs form heterodimers with RXR and bind to direct repeats.

Examples from this family include thyroid receptor α/β (TRα/β, NR1A1/2), vitamin D

receptor (VDR, NR1I1), retinoic acid receptor α/β/γ (RARα/β/γ, NR1B1/2/3), and

peroxisome proliferator-activated receptor α/β/γ (PPARα/β/γ, NR1C1/2/3). Unlike type I

NRs, type II NRs are constitutively in the nucleus. Without ligands, the NR heterodimers

bind to DNA with corepressor complexes and actively repress the expression of target genes.

When ligands are present, the binding of ligands changes the conformation of NR

heterodimers and leads to the expression of target genes. Some NRs like RAR-related

orphan receptor α/β/γ (RORα/β/γ, NR1F1/2/3) and steroidogenic factor 1 (SF-1, NR5A1) can

bind DNA as monomers. NRs that act as monomers and NRs that bind to direct repeats as

homodimers are classified as the other two classes (1).

1.1.2 Nuclear Receptors and Co-regulatory Proteins

NR-mediated gene expression is a dynamic process, which is rigorously controlled by a

series of exchanges between NRs and co-regulatory proteins. Both activation and repression

of NRs require the cooperation of co-regulatory proteins. In general, without ligands, NRs are associated with corepressor complexes, which actively repress the expression of target genes. The most well-studied corepressor proteins are nuclear co-repressor (NCoR) and SMRT. Neither NCoR nor SMRT has intrinsic enzymatic activity, but they function as platform proteins to recruit complexes that contain chromatin-modifying enzymes like histone deacetylases (HDACs) and many other enzymes (16-19). By deacetylating histones, HDACs generate a condensed chromatin structure to repress transcription. When ligands are present, the binding of ligands leads to a conformational change of the LBD. The corepressor complexes then dissociate from NRs. After the corepressor complexes are cleared from the promoter, coactivators together with the basal transcriptional machinery can be recruited to NRs to initiate the transcription of target genes (20). Steroid receptor coactivators (SRCs) are among the best characterized coactivators. They act as bridging factors between NRs and other co-regulators like histone acetyltransferases (HATs) through protein-protein interactions. Corepressor complexes and coactivators are large multiprotein complexes. Components in these complexes may have different enzymatic capabilities including acetyltransferase, methyltransferase, phosphokinase and ATPase activities. Both corepressor complexes and coactivators bind to the hydrophobic groove formed by α-helices on the surface of LBD. Because of the overlapping of binding sites, binding of corepressor complexes and coactivators are mutually exclusive.

To date, hundreds of co-regulatory proteins have been identified. As essential components of NR signaling, co-regulatory proteins regulate chromatin modifications, mediate crosstalk between different NRs, and contribute to cell-specific transcriptional

responses to a given receptor (21). Co-regulatory proteins are under the regulation of signaling pathways, and their activities are directly influenced by their post-translational modification (PTMs) status. For example, phosphorylation of SRC-3 on different residues can lead to selective activation of certain NRs and non-NR transcription factors (22). Phosphorylation of NCoR and SMRT can regulate their intracellular localization (23). The transcriptional activities and transcription factor preferences of co-regulatory proteins can be different in disparate cell types and signaling contexts. Sometimes the distinction between coactivators and corepressors can be blurred. They can even switch roles under certain conditions (24, 25). Misexpression and malfunction of co-regulatory proteins have been shown to be associated with numerous physiological abnormalities and diseases, including type II diabetes, cancer and some inherited genetic syndromes (26). With the increasing understanding of co-regulatory proteins, they have begun to gain attention as potential therapeutic targets.

1.1.3 Post-Translational Modifications of Nuclear Receptors

Activities of NRs are mainly regulated by their ligands and co-regulatory proteins, but their functions can also be influenced by PTMs. PTM is a process in which amino acid residues in a protein are covalently modified by other molecules or proteins. PTMs, like phosphorylation, SUMOylation, and acetylation, fine-tune the activities of NRs through the regulation of NR subcellular localization, dimerization, DNA binding, and co-regulator interactions. Potentially, every NR can be regulated by PTMs. Recently, PTMs of NRs have drawn much attention and interesting research has emerged.

1.1.3.1 Phosphorylation of NRs

The most extensively studied PTM of NRs is phosphorylation. Most NRs are phosphorylated at multiple sites in both ligand-dependent and –independent manners. Phosphorylation regulates transcriptional activities of NRs and provides a way to integrate the physiological context of cells and activities of NRs. In general, phosphorylation is a positive regulator for NR activities, though in some cases, it can also lead to transcriptional repression.

For instance, cyclin-dependent kinase 9 (CDK9) together with other CDKs can phosphorylate AR on S81. Treatment of CDK9 inhibitors decreases S81 phosphorylation and also AR-mediated transcription (27). Growth factors can activate ERα in a ligand-independent manner through the phosphorylation of ERα (28). For GRα phosphorylation, both the pattern and extent are influenced by different glucocorticoids presented (29). Different phosphorylation patterns can lead to different effects on GRα transcriptional activity. Phosphorylation at S211 leads to increased transcriptional activity, whereas phosphorylation at S226 leads to decreased transcriptional activity (30, 31). Moreover, stress-activating stimuli can phosphorylate GRα in a ligand-independent manner at S134 and lead to a blunted transcriptional response of select genes (32). Phosphorylation of PRs by MAPKs (mitogen-activated protein kinases) leads to ultra-sensitivity of PRs to one of its ligands, progestins (33).

Since phosphorylation can regulate the activities of NRs and lead to different transcriptional outcomes, phosphorylation of NRs has emerged as a potential therapeutic target. For example, phosphorylation of GR by p38 MAPK can lead to reduced

responsiveness to glucocorticoid treatment in patients with asthma. p38 MAPK inhibitors have been shown to increase the anti-inflammatory effects of glucocorticoids and re-establish the beneficial effects of glucocorticoids in glucocorticoid-resistant asthma (34). CDK5 phosphorylates PPARγ at S273 and decreases PPARγ transcriptional activity. Blocking CDK5-mediated PPARγ phosphorylation by non-agonistic binding has been demonstrated to produce anti-diabetic effects without some side effects of thiazolidinediones, suggesting that targeting the CDK5 phosphorylation event, instead of direct PPARγ agonism, may be a better way to treat insulin resistance and avoid side effects (35).

1.1.3.2 Acetylation of NRs

Protein acetylation is closely related to transcription.   Histone acetyltransferases (HATs) and histone deacetylases (HDACs) modify histones by adding or removing acetyl groups, generating either loosened or condensed chromatin structure to facilitate or repress transcription. Besides its role in regulating chromatin structure, acetylation also directly modifies NRs and regulates their activities.

Depending on target NRs, acetylation can either enhance or inhibit their transcriptional activities. For instance, mutation of acetylation sites of AR leads to increased cytoplasmic localization and decreased transcriptional activity of AR in PC3 cells. Inhibition of deacetylase activities increases acetylation level of AR and also its transcriptional activities (36, 37). PR can be acetylated at K183 by p300 and p300-mediated acetylation leads to increased PR activity (38). GR becomes acetylated in response to glucocorticoid binding. Unlike the NRs discussed above, acetylation of GR by the circadian rhythm-related

transcription factor "clock" represses GR activities (39).   Deacetylation of GR by HDAC2 is reported to be essential for the receptor to efficiently repress NF-κB (nuclear factor kappa-light-chain-enhancer of activated B cells) activity, suggesting that acetylation of GR limits the inhibitory actions of glucocorticoids on NF-κB signaling (40).

1.1.3.3 Ubiquitination of NRs

Ubiquitination is a small covalent modification that has been identified on many NRs. Ubiquitination can lead to proteasome degradation of NRs.   However, the regulatory effect of ubiquitination on activities of NRs is achieved through both degradation-dependent and -independent manners.

Polyubiquitination of AR can be catalyzed by ubiquitin E3 ligases MDM2 (Mouse double minute 2 homolog) and CHIP (C-terminus of Hsc70 interacting protein).   MDM2 and CHIP-mediated polyubiquitination of AR promotes its degradation and decreases AR transactivation (41, 42).   Ubiquitination of AR can be mediated by the ubiquitin E3 ligase RNF6 (RING finger protein 6) as well.   It has been shown that ubiquitination of AR is increased after ligand treatment, while knockdown of RNF6 significantly diminishes both basal and ligand-induced ubiquitination of AR.   Unlike MDM2 and CHIP, RNF6-promoted AR ubiquitination does not lead to its degradation, but enhances the transcriptional activity of AR (43).   Ubiquitination of GR has been shown to target the receptor for proteasome degradation and decrease GR transactivation (44).   Mutation of the GR ubiquitination site leads to resistance to ligand-dependent down regulation and potentiates transcriptional activities on glucocorticoid-responsive reporter genes (45).   All forms of PPARs have been

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shown to be ubiquitinated.   Polyubiquitination of PPARβ and PPARγ inhibits their activities by promoting their degradation.   However, the scenario is much more complicated for PPARα.   The ubiquitination of PPARα is promoted by the E3 ligase, MDM2.   In transient transfection assays, when the MDM2-to-PPARα ratio is less than 0.5, ubiquitination leads to increased activity of PPARα.   However, when the MDM2-to-PPARα ratio is greater than 1.0, ubiquitination inhibits transactivation of PPARα (46).   Another example of ubiquitinated NRs is PR.   Inhibition of proteasome activity prevents receptor degradation and suppresses PR-dependent transcription through failed recruitment of RNA polymerase II (47).   BRCA1 (breast cancer 1, early onset) inhibits the transcriptional activity of PR through its ubiquitin E3 ligase activity.   BRCA1 can regulate the ubiquitination and degradation of PR in the absence of hormones and have a direct effect on the cellular level of PR, which might explain why mutations of BRCA1 exert tissue specificity in preferentially elevating the risk of breast cancer (48).

1.1.3.4 SUMOylation of NRs

SUMO is the acronym of Small Ubiquitin-like Modifier.   Even though amino acid sequences of SUMO and ubiquitin are quite different, the structure of SUMO protein is similar to ubiquitin and the process of SUMOylation is comparable to ubiquitination. Recently, increasing numbers of NRs have been identified as SUMOylation substrates.   In general, SUMOylation is correlated with transcriptional repression.   The repression is achieved through recruitment of corepressor complexes.   Only in very few instances, SUMOylation leads to increased transcriptional activity.

AR is the first NR shown to be SUMOylated. Mutation of SUMOylation sites in AR results in enhanced transcriptional activities of AR in reporter gene assays (49). Recent studies using genome-wide gene expression analyses suggest that SUMOylation does not simply repress AR activities. Sutinen *et al.* showed that SUMOylation regulated the interaction between AR and chromatin, and contributed to selective target gene expression (50). Moreover, AR can directly regulate the expression of SENP1 (Sentrin-specific protease 1) through the androgen response element in SENP1 promoter (51). SENP1 is a member of the sentrin protease family, which plays dual roles in SUMOylation process. SENPs can facilitate SUMO maturation and also deconjugate SUMO from substrates. The mutual regulation between AR and SUMOylation pathway indicates the close involvement of SUMOylation in NR activities. GR is also post-translationally modified by SUMO and the SUMOylation level is increased by the binding of glucocorticoids (52). Depending on the site of SUMOylation, the transcriptional activities of GR can be either enhanced or repressed through alterations in the recruitment and/or activity of specific co-regulators (53, 54). Recent research indicates that SUMOylation regulates the activities of GR in a target locus-selective manner and affects genes both up- and down- regulated by GR (55). SUMOylation of PPARα in the hinge region and PPARγ in the AF-1 region block their transcriptional activities, possibly by promoting corepressors recruitment (56, 57). SUMOylation of PPARγ in the LBD results in its recruitment to promoters of inflammatory genes. SUMOylated PPARγ inhibits the expression of inflammatory genes by preventing clearance of corepressor complexes (58). With regard to PR, the SUMOylated receptor is

exceptionally stable and has repressed transcriptional activity, while deSUMOylated PR is

rapidly downregulated and transcriptionally hyperactive (59).

1.1.3.5 The Crosstalk between Different PTMs

PTMs on NRs are more complex than discussed above. NRs can also be modified by

methylation, Neddylation, and many other PTMs. Orchestration of these modifications is

required for full control of NRs activities. The effects of PTMs on NRs activities are also

dependent on physiological conditions, cell and tissue types, and specific genes being

regulated. These complexities may explain how tissue- and gene-specific regulations are

achieved in the same organism, by the same receptor protein and hormone.

The crosstalk between different PTMs can be sequential. The presence of one

modification can lead to or facilitate another modification. For example, both

phosphorylation-dependent SUMOylation and phosphorylation-dependent ubiquitination have

been identified (60, 61). Heterologous SUMO2/3-ubiquitin chains have also been observed.

It is suggested that SUMOylation by SUMO2/3 but not SUMO1 promotes ubiquitination and

ubiquitination-dependent degradation by the proteasome (62). A class of ubiquitin E3

ligases, including RNF4 (RING finger protein 4), are identified as SUMO-target ubiquitin

ligases, which are ubiquitin E3 ligases specifically recognize SUMOylated proteins to

facilitate ubiquitination on SUMOylated proteins (63). Heterologous SUMO2/3-ubiquitin

chains play important physiological and pathophysiological roles. One example is the

regulation of NF-κB activity. Under conditions of deficient SUMOylation, a delay in NF-κB

dependent transcription is observed (64). Another example involves acute promyelocytic

leukemia (APL). Arsenic is used to treat APL patients, since it can promote the proteasomal degradation of PML (promyelocytic leukemia) protein and the oncogenic PML-RARα fusion protein. Arsenic treatment first leads to increased SUMO2/3 modification of these proteins. RNF4, the SUMO-dependent ubiquitin E3 ligase, recognizes these SUMOylated proteins and targets these proteins for RNF4-mediated ubiquitination and degradation (65, 66). Data generated in our laboratory indicate that the presence of SUMO proteins (both SUMO1 and SUMO3) increases not only the ubiquitination of PXR but also the global ubiquitination. Though the underlying mechanism and the biological function remain unclear, it is feasible that the modification by SUMO can facilitate the ubiquitination of different substrates. Future efforts to identify the SUMO-dependent ubiquitin E3 ligases involved in this process and then knockdown of the identified ligases will further confirm the hypothesis.

In addition to sequential crosstalk, there is also spatial crosstalk among PTMs, like competition for the same site by different PTMs. The competition for the same modification sites suggests mutual exclusivity of these modifications. One instance is the competition between SUMO1 and ubiquitin for the same lysine in IκBα, which is an inhibitor for NF-κB. SUMOylation by SUMO1 inhibits the ubiquitination and degradation of IκBα, thus inhibits the activation of NF-κB pathway (67). Besides the competition for the same amino acid, crosstalk among adjacent sites in the primary amino acid sequence or higher order structure of protein also exists. For example, acetylation of FXR at K217 inhibits its SUMOylation at K277, partly by blocking the interaction with PIASy (protein inhibitors of activated STAT), which is the SUMO E3 ligase that promotes FXR SUMOylation (68).

However, the crosstalk between PTMs is much more complicated than discussed above. It is well-accepted that different forms of PTM interact with one another in a highly complex manner to control protein function. One modification can also indirectly influence other modifications. For instance, the ubiquitin conjugating enzyme E2-25K is inactivated by SUMOylation. SUMOylation of E2-25K hampers its interaction with the upstream E1 activating enzyme and inhibits ubiquitin thioester formation (69). Another example is MDM2, which is the major ubiquitin E3 ligase that regulates the stability and activity of p53. MDM2 not only facilitates ubiquitination of other proteins, including p53 and AR, but also its self-ubiquitination. SUMOylation of MDM2 prevents self-ubiquitination and hence, stabilizes MDM2 and increases its E3 ubiquitin ligase activity towards p53 (70, 71). Moreover, acetylation of MDM2 down-regulates its E3 ligase activity towards both p53 and Mdm2 itself (72). Furthermore, SUMOylation of Psmd1, a subunit of the proteasomal 19S regulatory particles, can alter proteasome composition and function, and impact the degradation of proteasomal targets, thus increases the accumulation of ubiquitinated proteins (73).

Different types of stresses can result in different sets of PTMs and lead to different activities of NRs. The studies on crosstalk of PTMs on NRs have just begun to emerge. Targeting PTMs can be a novel way to treat NRs-related diseases. Moreover, it should be noted that co-regulatory proteins of NRs can also be post-translationally modified, which represents another level in the regulation of NRs activities.

1. 1. 4 Liver-enriched Nuclear Receptors and Inflammation

Inflammation is the body's protective attempt to remove injurious stimuli, like foreign bacteria and viruses, and to initiate the healing process to restore tissue homeostasis. Once stimuli are successfully removed from the body, acute inflammation should be resolved soon. The initiation of acute inflammation has beneficial effects to protect the body. However, chronic inflammation is associated with many diseases. Consequently, metabolic syndrome and related disorders including obesity, atherosclerosis, and diabetes are all related to inflammation. Thus, it is of great importance to regulate the inflammatory response. Many NRs have been shown to repress inflammation and immune responses through diverse mechanisms. Glucocorticoids have been widely used for decades to treat inflammation related diseases. The liver is the major organ of acute phase protein synthesis. On the other hand, the liver is also the major organ for metabolism. It is the obvious target tissue for integrating metabolic signaling and inflammation. A number of publications indicate crucial roles of liver-enriched NRs in repressing inflammatory processes. Liver X receptors (LXRs, NR1H), peroxisome proliferator activated receptors (PPARs, NR1C) and farnesoid X receptor (FXR, NR1H4) have all been shown to repress inflammation.

LXRs inhibit the expression of inflammatory response genes through protein-protein interactions, where ligand bound LXRs become SUMOylated by SUMO2/3 and are recruited to inflammatory response gene promoters to inhibit the clearance of corepressors (74, 75). Other mechanisms have also been proposed. Canavan *et al.* showed that in dendritic cells, activation of LXRs prevented the nuclear translocation of the p50 subunit of NF-κB, thus inhibiting NF-κB activities (76). Li *et al.* showed that in macrophage, LXR activators

attenuated both DNA binding and transactivation potential of STAT1 (Signal Transducers and

Activators of Transcription 1) in response to IFN-γ treatment (77).

PPARs can attenuate inflammatory response through both direct and indirect

mechanisms. PPARα upregulates the expression of IκB, which tethers NF-κB in the

cytoplasm and functions as a negative regulator of NF-κB (78). The interleukin-1 receptor

antagonist (IL-1Ra), an anti-inflammatory cytokine that inhibits the IL-1 signaling pathway,

is a direct target gene of PPARα in the liver. Through upregulating IL-1Ra level, activation

of PPARα can decrease the activities of IL-1 signaling (79). Via activating AMPK

(AMP-activated protein kinase), PPARβ/δ can prevent ER stress, inflammation and insulin

resistance in skeletal muscle cells (80). PPARγ has been shown to prevent the removal of

corepressor complexes from the promoter of inflammatory response genes to inhibit their

expression (58).

The anti-inflammatory effect of FXR is demonstrated by the fact that FXR null mice

display strong hepatic inflammation. Activation of FXR inhibits NF-κB-mediated hepatic

inflammatory response but does not suppress NF-κB-activated anti-apoptotic genes in

primary mouse hepatocytes. It is suggested that FXR activation suppresses NF-κB

transcriptional activity by decreasing the binding between NF-κB and DNA sequences (81).

The loss of FXR also leads to exacerbated inflammation in rodent models of colon

inflammation. Consistent with the findings, the expression level of FXR is reduced in both

patients with Crohn's disease and rodent models of colitis. The protective role for FXR is

further confirmed by the fact that activation of FXR by ligand treatment protects against the

development of colitis in wild type mice but not in FXR-KO mice (82). The underlying

mechanism is suggested to involve SUMOylation of FXR, since mutation of the potential

SUMOylation site impairs the transrepression activity of FXR (82).

In addition to the NRs discussed above, anti-inflammatory properties have also been

found for other liver-enriched NRs. Small heterodimer partner (SHP, NR0B2) has been

suggested to be an intrinsic negative regulator of Toll-like receptor (TLR)-triggered

inflammatory response, acting as both a repressor of the NF-κB subunit p65 and an inhibitor

of ubiquitination of TRAF6 (83). Moreover, SUMOylated LRH1 (liver receptor homolog-1,

NR5A2) can prevent the removal of co-repressor complexes from the promoter of

inflammatory response genes (84).

1.2. Pregnane X Receptor

1.2.1 General Remarks

Discovered in 1998 based on the structural homology to other NRs, PXR gets its name

from the activation by pregnane (21-carbon or C_{21}) steroids (85). It was considered as an

orphan nuclear receptor until xenobiotics were identified as PXR ligands. Nowadays, PXR

is well-known as the xenobiotic sensor to increase the metabolism and clearance of drugs and

xenobiotics from the body, and protect the body from potential toxic insults. It is a member

of the NR1I subfamily, which also contains vitamin D receptor (VDR, NR1I1) and

constitutive androstane receptor (CAR, NR1I3). PXR is most closely related to VDR in the

NR family, sharing 64% identity in the DBD and 39% in the LBD (86). However, CAR has the most overlapping ligand specificity and function with PXR. With overlapping but distinct functions, these NRs work both individually and collaboratively to regulate the metabolism and elimination of both endogenous and exogenous compounds (87).

PXR forms a heterodimer with RXRα and binds to PXR response elements to regulate the expression of its target genes. Consistent with its function in xenobiotic clearance, PXR is highly expressed in the major detoxifying organs such as liver and intestinal tract, but low levels of expression can also be found in other tissues, like kidney and stomach. PXR is conserved through evolution within the animal world and has been cloned and functionally expressed from zebrafish, frog, chicken, and multiple mammalian species.

The most remarkable characteristic of PXR is the promiscuity with respect to ligands. PXR can be activated by xenobiotics, steroid hormones, and bile salts among many compounds with diverse structures. In point of fact, PXR has the broadest ligand specificity among the NR family. The promiscuity of PXR ligands is related to the unique binding capability of its LBD. The ligand binding cavity of PXR is large, smooth, and hydrophobic, unlike 'typical' NR LBDs, which compact ligand-binding cavities to the approximate shape of specific ligands (88). The PXR ligand-binding cavity also shows considerable flexibility to accommodate the binding of structurally-diverse ligands. One interesting fact is that just like PXR, proteins encoded by PXR target genes, like CYP3A4 (Cytochrome P450 3A4) and P-glycoprotein, also show substrate promiscuity, and can metabolize or bind to a diverse class of chemically unrelated compounds.

Besides ligand promiscuity, another important feature of PXR is the species difference. Ligands for PXR are quite diverse among different species. For example, PCN (Pregnenolone 16α-carbonitrile) is a strong murine PXR agonist, but it has little effect on human PXR. Rifampicin is a potent human PXR ligand that has less effect on murine PXR. The sequence divergence in PXR LBD among different species is suggested to be responsible for the species difference. The sequence similarities of PXR LBD across species are the lowest in the NR family, with other NRs at least 10-15% higher (11). Besides species difference in ligands, signaling pathways can also have different regulatory effects on PXR function in different species. For instance, while activation of the cyclic AMP-dependent protein kinase signaling pathway increases PXR-mediated gene activation in mouse hepatocytes, the same signaling pathway represses PXR-mediated gene activation in rat and human hepatocytes (89). It is suggested that the species difference of PXR is to accommodate the needs of different species. Since different species encounter different compounds, the profiles of xenobiotics that the zebrafish would encounter are definitely not the same as rodents would encounter.

1.2.2 PXR in Xenobiotic Metabolism and Clearance

PXR activation protects animals against potentially toxic compounds by facilitating their metabolism and clearance (90). Upon activation by its ligands, PXR up-regulates the expression of genes that encode detoxification enzymes and transporters. The protective role of PXR can be proved by the findings that after being challenged with toxic compounds

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such as lithocholic acid (LCA), PXR-KO mice display much severe liver damage compare to wild type mice (91).

Activation of PXR leads to increased expression of phase I (oxidation) and phase II (conjugation) metabolizing enzymes and phase III transporters to enhance the metabolism and transport of a broad range of endogenous and exogenous compounds. The phase I metabolizing enzymes facilitate the oxidative metabolism of endogenous substances and xenobiotics. Cytochrome P450 enzymes convert lipophilic compounds into more hydrophilic products, making them ready for further biotransformation by phase II enzymes (92). CYP3A4, one of the well-characterized PXR target genes, is the most abundant CYP expressed in human liver. While PXR has a broad ligand specificity, CYP3A4 also has a remarkably broad substrate specificity, and metabolizes more than half of the pharmaceuticals on the market, which makes PXR activation closely related to adverse drug events (93).

Through conjugation, phase II drug metabolism enzymes greatly enhance the water solubility of compounds containing conjugatable groups that are either present on the compounds or introduced by phase I metabolizing enzymes, and promote their excretion. Activation of PXR can increase the expression of phase II drug metabolism enzymes like uridine-5′-diphosphate glucuronosyl transferase (UGT), sulfotransferase (SULT), and glutathione S-transferase (GST) to facilitate the excretion of toxic compounds (94-97).

In addition to its role in regulating metabolism enzymes, PXR also regulates drug transporters for efflux and uptake of endogenous and exogenous compounds (98). Some examples of drug transporters that are regulated by PXR are MDR1 (multidrug resistance

protein 1), MRPs (multidrug resistance proteins), and OATP2 (organic anion transporting protein 2). (99, 100). Up-regulation of these proteins by PXR provides a critical determinant of the bioavailability of drugs in the body. PXR activation is suggested to be responsible for drug resistance in cancer cells.

PXR activation protects the body from exposure to toxic compounds. However, activation of PXR is not without a risk. As a key regulator of drug metabolism and clearance, unexpected or abnormal activation of PXR may lead to adverse drug-drug and disease-drug interactions, which are major clinical problems increasing not only the medical costs but also morbidity and mortality. Altered levels of CYP3A4 and MDR1 can significantly affect the therapeutic response of a variety of administered drugs, and can cause serious drug-drug interactions (101). PXR-dependent adverse drug-drug interactions may be avoided if suitable PXR antagonists are widely available.

1.2.3 Other Biological and Pathophysiological Functions of PXR

1.2.3.1 Glucose Homeostasis

Hepatic gluconeogenesis is required for survival during prolonged fasting or starvation. Activation of PXR has been suggested to suppress gluconeogenesis, mostly through protein-protein interactions. PXR can repress glucagon-activated transcription of *G6Pase* (glucose-6-phosphatase) and *PEPCK* (phosphoenolpyruvate carboxykinase). Both enzymes play key roles in the homeostasis of blood glucose level. PXR mediated suppression is achieved through the interaction with CREB [CRE (cAMP-response element)-binding

protein], which is the transcription factor that activates the transcription of G6Pase and PEPCK. PXR binds directly to CREB and this binding prevents the interaction between CREB and CRE (102). FOXO1 (Forkhead box protein O1) is another activator of gluconeogenic genes. PXR directly interacts with FOXO1 to prevent its binding to insulin response sequence (103). The interaction between PXR and FOXO1 is enhanced by PXR ligand. Through the inhibition of FOXO1, PXR suppresses gluconeogenesis. HNF-4 is another activator for *G6Pase* and *PEPCK* expression. Bhalla *et al.* suggested that ligand-activated PXR attenuated HNF-4 signaling by competing for the coactivator PGC-1 (Peroxisome Proliferator Activated Receptor Gamma Co-activator 1), and resulted in repressed expression of *G6Pase* and *PEPCK* (104). In support of this suggestion, PCN treatment decreases blood glucose levels in fasting wild-type, but not in PXR-KO mice (105).

1.2.3.2 PXR in Lipid Homeostasis

Both genetic (using the VP-hPXR transgene) and pharmacological (using the PXR agonist) activation of PXR have been shown to induce hepatic steatosis, suggesting the regulatory role of PXR in lipid homeostasis (106-108). Activation of PXR can increase *de novo* lipogenesis, increase fatty acid uptake, and at the same time, inhibit fatty acid β-oxidation. All of these processes contribute to the pathogenesis of steatosis.

S14 protein can transduce hormone-related and nutrient-related signals to genes involved in lipid metabolism and plays an important role in the induction of lipogenic enzymes (109, 110). By regulating the expression of *S14* (thyroid hormone–responsive SPOT14 homolog), PXR contributes to *de novo* lipogenesis (111). PXR can also directly bind to *CD36*

promoter to regulate *CD36* expression, which is the transporter that transports free fatty acids into cells (112). Increased expression of CD36 leads to enhanced fatty acids uptake and hepatic lipid accumulation. β-oxidation is an important step of fatty acid catabolism. The expression of *PPARα* and *thiolase*, both of which regulate β-oxidation, is decreased by PXR, suggesting that activation of PXR suppresses β-oxidation (108). Cpt1a (carnitine palmitoyltransferase 1A) and Hmgcs2 *(*mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2) are two enzymes regulating β-oxidation and ketogenesis. FoxA2 is the key regulatory factor that activates the transcription of these genes in fasting mouse liver (113). PXR could bind to FoxA2 and prevent the binding of FoxA2 to the promoter of target genes, thus inhibiting the expression of *Cpt1a* and *Hmgcs2* (105). By simultaneously increasing *de novo* lipogenesis, increasing fatty acid uptake and inhibiting fatty acid β-oxidation, PXR contributes to the accumulation of lipids in cells.

1.2.3.3 PXR and Bile Acids Homeostasis

The conversion of cholesterol to bile acids occurs exclusively in hepatocytes. Cholesterol 7-α-hydroxylase, also known as cytochrome P450 7A1 (CYP7A1), regulates the first and rate limiting step in bile acids synthesis. PXR together with many NRs tightly control the level of bile acids in the body through a complex network. While bile acids are endogenous ligands for FXR, PXR can also be activated by bile acids (91, 114). Upon activation, PXR induces the expression of its target genes, including *CYP3A* and *OATP2*. While CYP3A can catalyze side-chain hydroxylation to make bile acids more hydrophilic, and promote their excretion, OATP2 facilitates the transport of bile acids, increasing the

uptake and excretion of them. Besides positively regulating gene expression, activation of

PXR also inhibits the expression of *CYP7A1* through its interaction with HNF-4α. HNF-4α

is a positive regulator of CYP7A1 expression. The interaction with PXR disrupts

HNF-4α/PGC-1α interaction and reduces the transcriptional activity of *CYP7A1* gene

promoter (115). Through the regulation of gene expression, PXR simultaneously regulates

the biosynthesis, transport, and metabolism of bile acids, indicating that PXR serves as a

physiological sensor not only for xenobiotics but also for bile acids. Because of this, PXR

has been proposed as a therapeutic target for the treatment of cholestasis (116).

1.2.3.4 PXR in Vitamin D Metabolism and Bone Mineral Homeostasis

It has been known for decades that long-term treatment with PXR activators can

decrease serum vitamin D levels and bone mineral density, leading to drug-induced

osteomalacia (117, 118). Calcium is a major component in bone development and

maintenance, whose absorption and excretion are regulated by vitamin D. Vitamin

D_3 24-hydroxylase (CYP24A1) converts the active 1α, 25-dihydroxyvitamin D3 (1, 25(OH)

2D3) into an inactive metabolite, acting as a feedback regulatory factor in calcium

homeostasis. Activation of PXR has been suggested to both activate and repress *CYP24A1*

expression (119, 120). It has been demonstrated that in the absence of vitamin D_3, PXR

could activate *CYP24A1* promoter by directly binding to the VDRE (Vitamin D response

element) region. However, in the presence of vitamin D_3, PXR indirectly bound to the

VDRE region and locked SMRT onto VDR to repress *CYP24A1* promoter. Moreover,

CYP3A4, whose expression is regulated by PXR, also metabolizes and inactivates 1α,

25-dihydroxyvitamin D3 (1, 25(OH) 2D3) (121, 122). Even though the affinity and efficiency of CYP3A4 to metabolize 1α, 25-dihydroxyvitamin D3 (1, 25(OH) 2D3) is lower than CYP24A1, the abundant expression level of CYP3A4 might make it the dominant player in 1α, 25-dihydroxyvitamin D3 (1, 25(OH) 2D3) metabolism in the liver.

1.2.3.5 PXR and Cancer Development

PXR is over-expressed in many cancer cells (123-125). The increased PXR activity leads to enhanced expression of drug metabolism enzymes and drug transporters, like CYP3A4 and P-glycoprotein, which increase drug clearance and decrease the exposure of cancer cells to anticancer drugs, thus leading to drug resistance. In addition to drug metabolism and clearance, PXR also regulates tumor proliferation and metastasis as well as apoptotic/anti-apoptotic processes. PXR is well-known to regulate hepatocytes proliferation and is required for normal progression of liver regeneration (126-128). Besides hepatocytes, PXR also regulates cell growth in different cancer tissues (129, 130). Additionally, PXR activation induces metastasis of cancer cells. It has been shown that activated PXR elicits p38 phosphorylation, which can lead to cell migration (131). PXR also induces metastasis of the primary human colon cancer tissues xenografted into immune-deficient mice. Moreover, PXR expression is reported to be higher in invasive stage than in early stage of breast cancer patients (132, 133). The activation of PXR can also increase cell viability and repress drug-induced apoptosis. PXR agonists can increase the expression of Bcl-2 and Bcl-xL, two apoptosis inhibitors, at both mRNA and protein levels in human and rat hepatocytes (134). Besides hepatocytes, PXR also inhibits

apoptosis in non-hepatic cancers, like colon cancer (135).   However, there are also many

incidents in which PXR induces cell apoptosis, suggesting that PXR-regulated cell

proliferation and apoptosis are tissue and cell/context-specific (136, 137).

Because of the roles of PXR in cancer development and progression, antagonists of PXR

has been proposed to improve the bioavailability of anti-cancer drugs and to reverse

PXR-mediated cancer drug resistance and tumor growth (138).   Controversy still remains for

the roles of PXR in cancer development, so further studies that decipher the function of PXR

in different cancers will be useful to therapeutically target PXR in PXR expressing cancers.

1.2.3.6. PXR and Inflammation

Long before the identification of PXR, it has been noticed that treatment of rifampicin,

later known as a prototypical human PXR ligand, can suppress humoral and cellular

immunological response (139).   The expression levels of inflammatory cytokines are higher

in hepatocytes from PXR-KO mice compared to wild type mice.   Activation of PXR

decreases the expression of inflammatory cytokines in isolated wild type hepatocytes but not

in PXR-KO hepatocytes, suggesting PXR-dependent repression in the inflammatory response

(140).

Besides in hepatocytes, similar effects were also observed in the intestine.   PXR has

been suggested as a therapeutic target to treat inflammatory bowel disease.   Through

inhibition of NF-κB target gene expression, PXR activation ameliorates dextran sulfate

sodium (DSS)-induced inflammatory bowel disease (141). Some drugs that have been used to treat inflammatory bowel diseases later turned out to be PXR ligands.

While the anti-inflammation property of PXR has been known for decades, the underlying mechanism is still vague. Our previous publication demonstrates that SUMOylation of PXR is essential for the repression of inflammatory genes (140). The role of PXR and SUMOylation in inflammation will be discussed in detail in chapter 3.

Humans are constantly exposed to xenobiotics that activate PXR. From the previous discussion, it is clear that PXR regulates both xenobiotic and endobiotic homeostasis. Besides the pathophysiological pathways discussed above, PXR also has regulatory effects on inflammation, bilirubin detoxification, steroid hormones and vitamin metabolism, among many more to be discovered. Therefore, PXR activation has important implication in many pathophysiological conditions. Recent studies indicate that activation of PXR is beneficial in the treatment of many diseases, like cholestasis, inflammatory liver disease and inflammatory bowel diseases, suggesting the potential of PXR as a therapeutic target. The plethora roles of PXR also raise the questions like how PXR is regulated to coordinate different biological pathways, how PXR function is balanced within these diverse pathways. Post-translational modifications such as phosphorylation have been shown to modulate the activity of many NRs, including PXR, and constitute an important mechanism for crosstalk between signaling pathways and NR-mediated gene expression. In the following chapters, some post-translational modifications of PXR and their effects on PXR function will be discussed in detail.

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Chapter 2: Identification of Novel PXR Interacting Proteins using Yeast Two Hybrid System

As discussed in chapter 1, PXR is an important regulator of xenobiotic metabolism and clearance. Through protein-protein interactions, PXR also regulates biological processes like glucose homeostasis, lipid homeostasis, and bile acid homeostasis. Since protein-protein interactions control the function of both PXR and its interacting proteins, the identification of novel PXR interacting proteins can lead to better understanding of the function and regulation of this important nuclear receptor. In this chapter, yeast two hybrid system was used to explore novel PXR interacting proteins in a human liver cDNA library.

2.1 Introduction to yeast two hybrid system

The yeast two hybrid system, developed by Fields and Song in 1989, is a widely-used method to study protein-protein interactions (1). This method is based on the properties of GAL4, which is a transcription factor that regulates the expression of enzymes for galactose metabolism in yeast. Like most transcription factors, there are two functional domains in GAL4: an N terminal DNA binding domain (DBD) and a C terminal activation domain (AD). The DBD can bind to the promoter of target genes in a sequence-dependent manner, but it cannot activate gene expression. The AD has activating regions, but it also cannot activate gene expression on its own, since it fails to bind to DNA. One important feature of transcription factors is that once the DBD and AD are in close proximity, even though they are not covalently attached to each other, a functional transcription factor can be reconstituted. Based on this characteristic, in the yeast two-hybrid system, one protein is fused to the DBD

of GAL4 (bait) and another protein is fused to the AD of GAL4 (prey). If these two proteins interact with each other, they will bring the DBD and AD together, thus restoring the function of GAL4, and this leads to the expression of one or more reporter genes in engineered yeast strains (illustrated in Fig. 2-1).

In the yeast two-hybrid system, the interactions between proteins are reflected by the expression of reporter genes. Fields and Song introduced GAL1-lacZ fusion gene into a GAL4/GAL80 null yeast strain (1). The GAL1 promoter contains DNA sequences to which the GAL4 protein binds. GAL80 is a negative regulator of GAL4, whose regulatory effect is dependent on galactose availability. In the absence of galactose, GAL80 binds to GAL4 and keeps it inactivated. When galactose is present, the inhibition of GAL80 on GAL4 is relieved, leading to the expression of GAL4 target genes (2). Since in engineered yeast strains, endogenous GAL4 and GAL80 are genetically deleted, the expression of lacZ solely depends on the function of exogenous GAL4: the GAL4 DBD and AD which are fused to the proteins of interest. By detecting the activity of β-galactosidase, protein-protein interactions can be examined. Since the invention of yeast two-hybrid system, numerous reporter genes and engineered yeast strains have been developed. In our experiment, we employed strain Y153, which is also a GAL4/GAL80 null yeast strain (3). It contains the GAL1-lacZ fusion gene introduced earlier, as well as a HIS3 reporter gene, which is also under the control of the GAL1 promoter. HIS3 encodes imidazoleglycerol phosphate (IGP) dehydratase, an indispensable enzyme for histidine biosynthesis. Theoretically, without protein-protein interaction yeast cells should not survive in histidine dropout medium (medium lacking

Figure 2-1

A
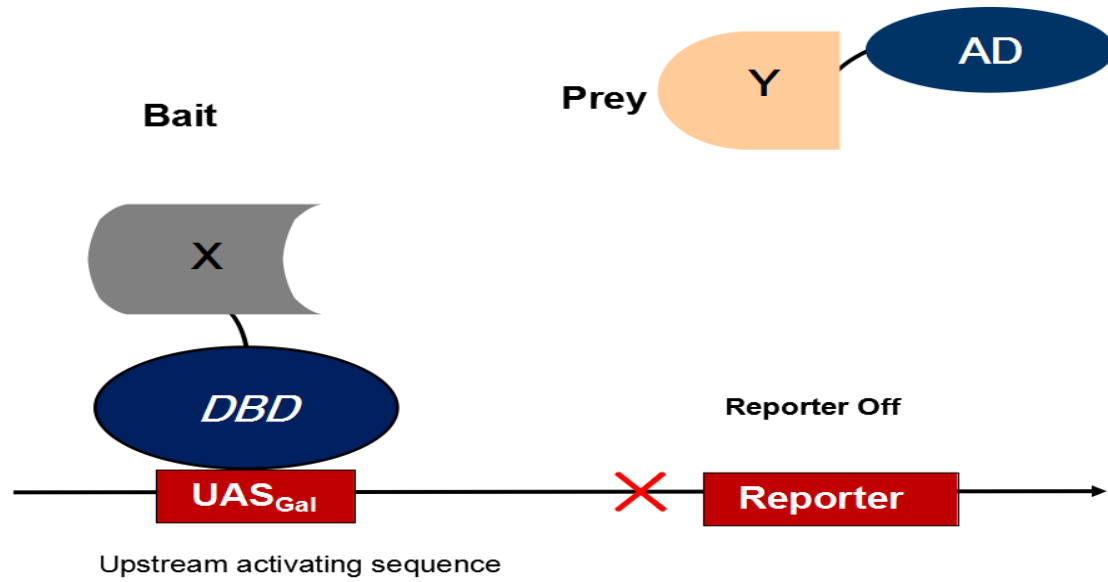
B
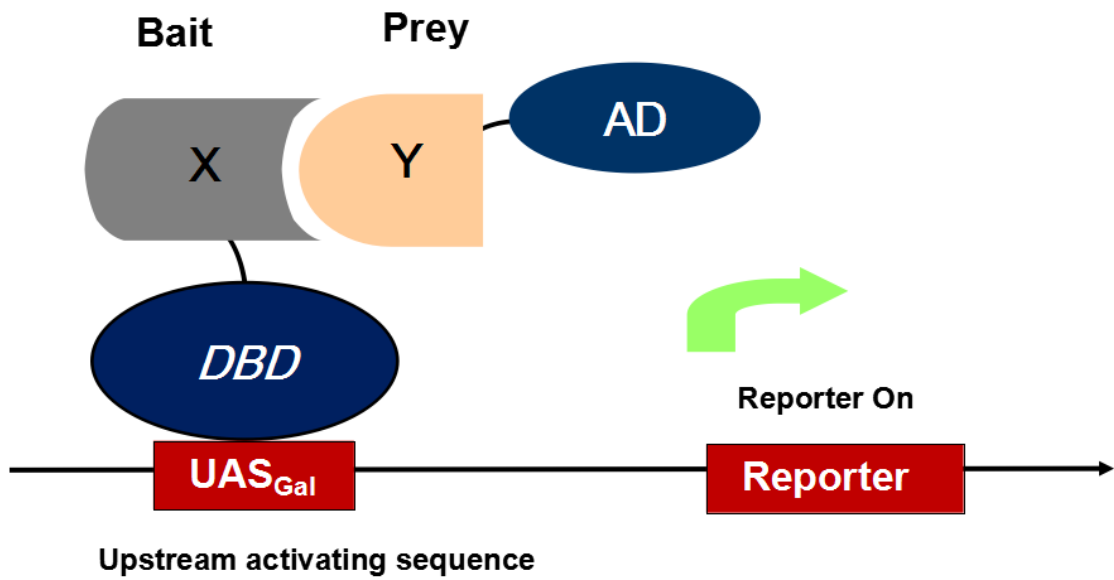
Figure 2-1.   Schematic Representation of Yeast Two Hybrid System.   (A) In the yeast two hybrid system, protein X is fused to the DNA binding domain (DBD) and this fusion protein is known as 'bait'.   Protein Y is fused to activation domain (AD) and this fusion protein is termed 'prey'.   If protein X and Y do not interact with each other, DBD and AD would be isolated from each other, the transcription of reporter gene cannot be initiated.   (B) If these two proteins interact with each other, they will bring the DBD and AD together, and restore the function of GAL4, leading to the expression of one or more reporter genes in engineered yeast strains.

histidine), since the essential amino acid cannot be produced without IGP dehydratase. However, the HIS3 reporter is relatively "leaky" and residual expression of the reporter is enough for yeast cells to grow even in the dropout medium.   In order to reduce false positive results, 3-aminotriazole (3-AT), an inhibitor of IGP dehydratase, is added to the medium to increase the stringency of the system.   Y153 also contains auxotrophic mutations for tryptophan and leucine, which can be complemented by plasmids encoding prey and bait proteins, and serves as an extra way to eliminate background growth.

In this chapter, we try to identify novel interacting proteins for PXR by utilizing the yeast two hybrid system to screen a human liver cDNA library.   After the potential PXR interacting proteins were identified, the results were further confirmed using another yeast stain Y190, and also in mammalian cells using a mammalian two hybrid system.

2.2 Materials and Methods

Plasmids.   Human PXR-LBD was generated by PCR from pSG5-hPXR using primers 5'-GAC GCC ATG GAG GGC ATG AAG AAG GAG ATG ATC-3' and 5'-GAC GCC GGA TCC CTA GCT ACC TGT GAT GCC GAA C-3' designed with NcoI and BamHI sites. The PCR product was subcloned in frame into GAL4 DNA binding domain vector pGBKT7 (Clontech) using NcoI and BamHI sites.   pACT2-hPXR-LBD was subcloned from pGBKT7-hPXR-LBD using NcoI and BamHI sites.   For pGBKT7-LBP4, LBP4 was excised from pACT2-LBP-4 with BglII and inserted into pGBKT7 multiple cloning site (MCS) using

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BamHI site. To build pM-LBP-4, LBP-4 was excised from pACT2 LBP-4 with BglII and then excised LBP-4 fragment was inserted into pM MCS using BamHI site. BamHI sites were introduced to SUG1 using primers 5'-GAC GGC GGG ATC CGT GGA CCA GAG CAG ATG GAG CTG GAG-3' and 5'-GAC GGC GGA TCC TCA TTA TTG GAG AGA TAC ACA CAA AGG-3', PCR product was subcloned into pM BamHI site to produce pM-SUG1. SMRT-ID2 was excised from pACT2-LBP227/SMRT-ID2 with BglII and inserted into pM BamHI site. The validity of all constructs was confirmed by sequencing.

Yeast Transformation, Cell Lysis and Western Blotting. Yeast were transformed using the lithium acetate (LiAc)-mediated method. In brief, both plasmid and carrier DNA were mixed with yeast competent cells with PEG/LiAc. After incubation, DMSO was added to the cell/DNA mixture before heat shock. After heat shock, cells were collected by centrifugation and resuspended in $1 \times$TE buffer (10mM Tris, pH 7.5, 1mM EDTA) and plated on appropriate selective plates. After colonies formed, a single colony was expanded in appropriate selective medium. After growth, yeast cells were harvested in complete cracking buffer (8M Urea, 5% SDS, 40 mM Tris pH 6.8, 0.1 mM EDTA, 0.4mg/ml Bromophenol blue, adding 1% β-mercaptoethanol, $1 \times$PIC before use) with glass beads. Whole cell lysates were subjected to SDS-PAGE and the gel was transferred to polyvinylidene difluoride membrane with subsequent western blot analysis using an anti-PXR antibody (sc-48340, Santa Cruz).

Yeast Two Hybrid Analysis. Y153 yeast cells were transformed with pGBKT7-hPXR-LBD construct encoding bait protein to screen a human liver cDNA library

constructed in vector pACT2 (Clontech).　Yeast cells were plated on selection plates lacking histidine, tryptophan and leucine and in the presence of 25mM 3-Aminotriazole (3-AT). Primary positive colonies were tested for LacZ expression by colony-lift filter assay.　Prey plasmids of positive colonies were recovered and sequenced.　The identities of the encoded putative interacting proteins were determined by data base search (Blastx).

Quantitative β-galactosidase Assay.　After growing overnight in the selection medium, yeast cells were transferred to complete medium to grow until the OD at 600 nm reached 0.5-0.8.　Cells were washed and resuspended in Z-buffer (60mM Na_2HPO_4, 60mM NaH_2PO_4, 10mM KCl, 1mM $MgSO_4$, pH 7.0) and then subjected to freeze (liquid nitrogen) and thaw (37 ℃ water bath) cycle to lyse cells.　Z-buffer containing 4mg/ml o-nitrophenyl-β-D-galactopyranoside was applied to cell lysates.　After the yellow color developed, reaction tubes were centrifuged to get the supernatant and subjected to measurement at an OD of 420 nm.　Assay results were normalized to the cellular absorbance value.

β-galactosidase units were calculated using the formula:

$$\beta\text{-galactosidase units} = 1000 \times OD420 / (t \times V \times OD600)$$

t=elapsed time of incubation

V=0.1ml×concentration factor

OD600= A600 of 1ml culture

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Transient Transfection and Mammalian Two Hybrid System. CV-1 cells were plated in 96-well plates at 7000 cells per well. The next day, the cells were transfected with the indicated plasmids using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. 24 hours post-transfection, cells were treated for another 24 hours with either vehicle or 10μM rifampicin. Luciferase activities were determined using a standard luciferase assay system (Promega). Luciferase assay results were normalized by β-galactosidase activities, which were determined by o-nitrophenyl-β-D-galactopyranoside assay.

2.3 Results

The Identification of Novel PXR Interacting Proteins in Yeast Two Hybrid System in Y153 Cells. Before the screening, the expression of bait protein, GAL4-DBD-hPXR-LBD, was confirmed in yeast cells (shown in Fig.2-2). It was further confirmed that the fusion protein did not autonomously transactivate the reporter genes. After these confirmations, Y153 cells were co-transformed with the construct encoding bait protein and the commercially available human liver cDNA library constructed in pACT2 vector (Clontech). After yeast colonies were formed on dropout (-Trp-Leu-His) plates supplied with 25mM3-AT, colonies were lifted and subjected to the X-gal test. The interaction was monitored by the appearance of blue color. Around 6 million colonies were screened and the colonies were named as LBPs (PXR-LBD-binding Proteins). The screening procedure is shown in Fig. 2-3.

Figure 2-2
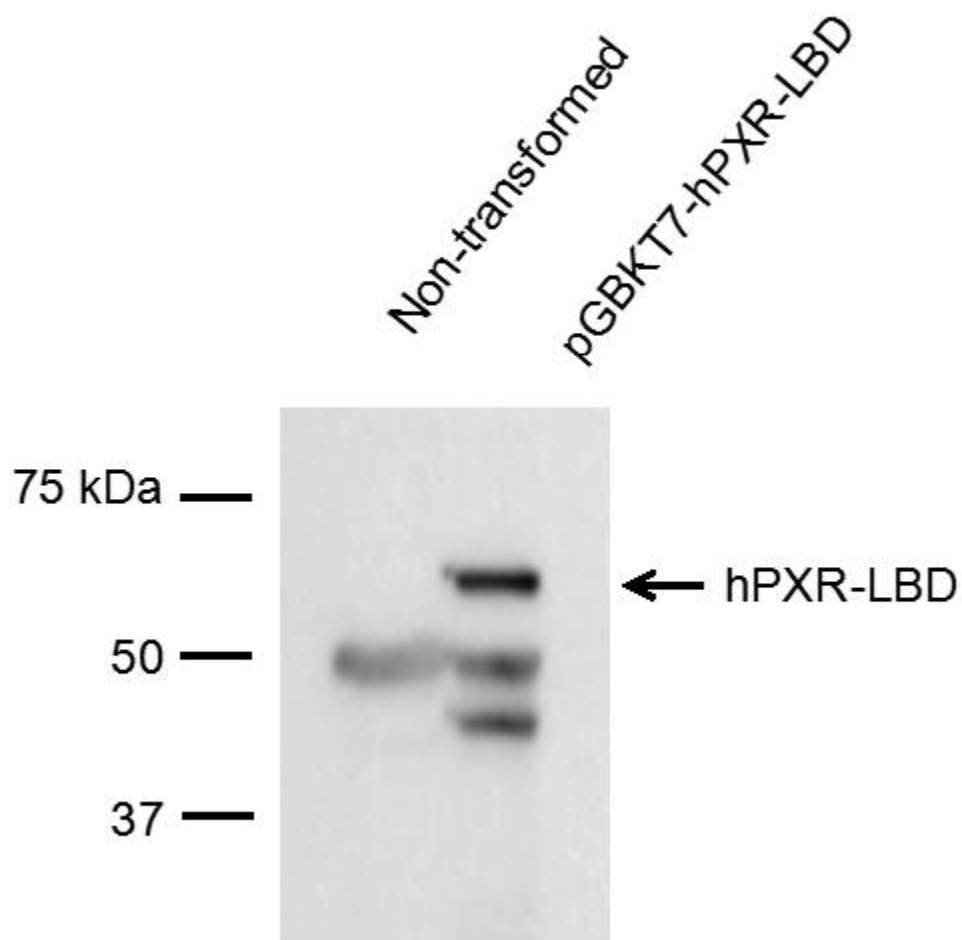
Figure 2-2. Expression of GAL4-DBD-hPXR-LBD in Y153 Cells. Y153 cells were transformed with pGBKT7-hPXR-LBD expression vector. After the colonies were formed, one single colony was picked and inoculated in tryptophan dropout medium. Non-transformed Y153 cells were grew on YPD plate and inoculated in YPD medium as control. After growth, yeast cells were harvested in complete cracking buffer. Whole cell lysates were subjected to SDS-PAGE and subsequent western blot analysis using an anti-PXR antibody (sc-48340, Santa Cruz).

Figure 2-3

Figure 2-3.   Overview of the Yeast Two Hybrid Screening for hPXR-LBD Interacting

Proteins.

Among all the colonies, 8 positive ones were chosen for further study.   The plasmids
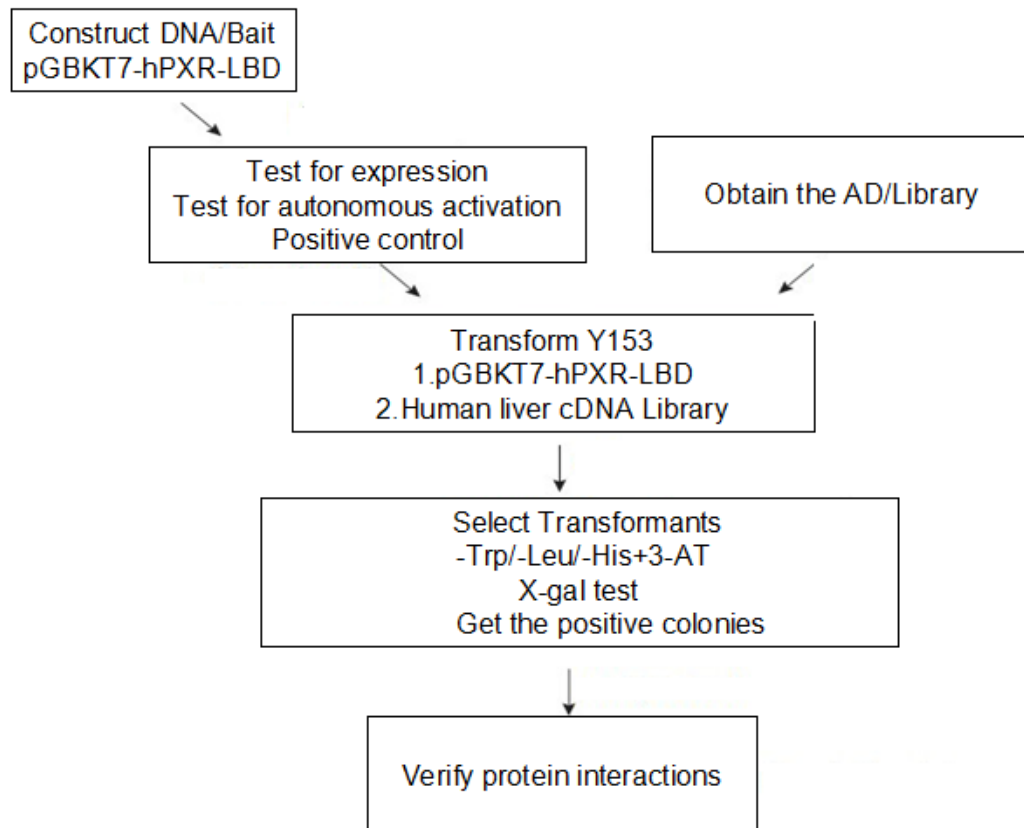
harbored by positive yeast colonies were rescued using E. coli, and then transformed back to

Y153 yeast cells together with pGBKT7-hPXR-LBD to further confirm the interaction.

There were two false positives.   The others were subjected to sequencing and the identities

of the encoded putative interacting proteins were determined by data base search (Blastx) and

were listed in Table 2-1.   Among them, SUG-1, SHP, and NCoR2/SMRT (hereinafter,

SMRT) are proteins which have already been recognized to interact with PXR (4-6).   The

identification of known PXR interacting proteins lends credit to the validity of our screening.

For the remaining two proteins, stromal cell derived factor 4 (SDF4) is localized in the Golgi

lumen (7), the subcellular compartment where PXR is believed not to localize under normal

circumstances.   Therefore, in the following experiments, we primarily focused on LBP-4.

LBP-4 is a fragment of the protein p0071, which is a member of Armadillo (ARM)

repeat-containing proteins.

Confirmation of the Interaction Between hPXR-LBD and LBP-4 in Yeast Cells.

Utilizing Y153 yeast cells, LBP-4 was identified as a potential hPXR-LBD interacting protein.

To test whether this interaction was specific to hPXR-LBD, other bait protein constructs

encoding NRs like Farnesoid X Receptor-ligand binding domain (FXR-LBD), Retinoid X

Receptor α-ligand binding domain (RXRα-LBD) and other constructs encoding non-related

bait proteins like O-acetyltransferase-related protein (OAT1) and organic cation transporter 1

(OCTN1) were employed to test their interactions with LBP-4.   Among all these bait

proteins, LBP-4 only interacted with PXR-LBD (as shown in Fig. 2-4).   This indicates that

the interaction between LBP-4 and hPXR-LBD is restricted between LBP-4 and PXR but

Table 2-1. Identities of the Identified hPXR-LBD Interacting Proteins from Yeast Two Hybrid Screening in Y153 Cells.

Number	Identity	
LBP-0	SDF-4	Stromal Cell Derived Factor 4
LBP-1	SUG-1	Suppressor for Gal 1
LBP-4	p0071	
LBP-22	SHP	Small Heterodimer Partner
LBP-226	hypothetical protein	
LBP-227	NCoR2/SMRT	Nuclear Receptor Co-repressor 2/ Silencing Mediator of Retinoic Acid and Thyroid Hormone Receptor

Figure 2-4
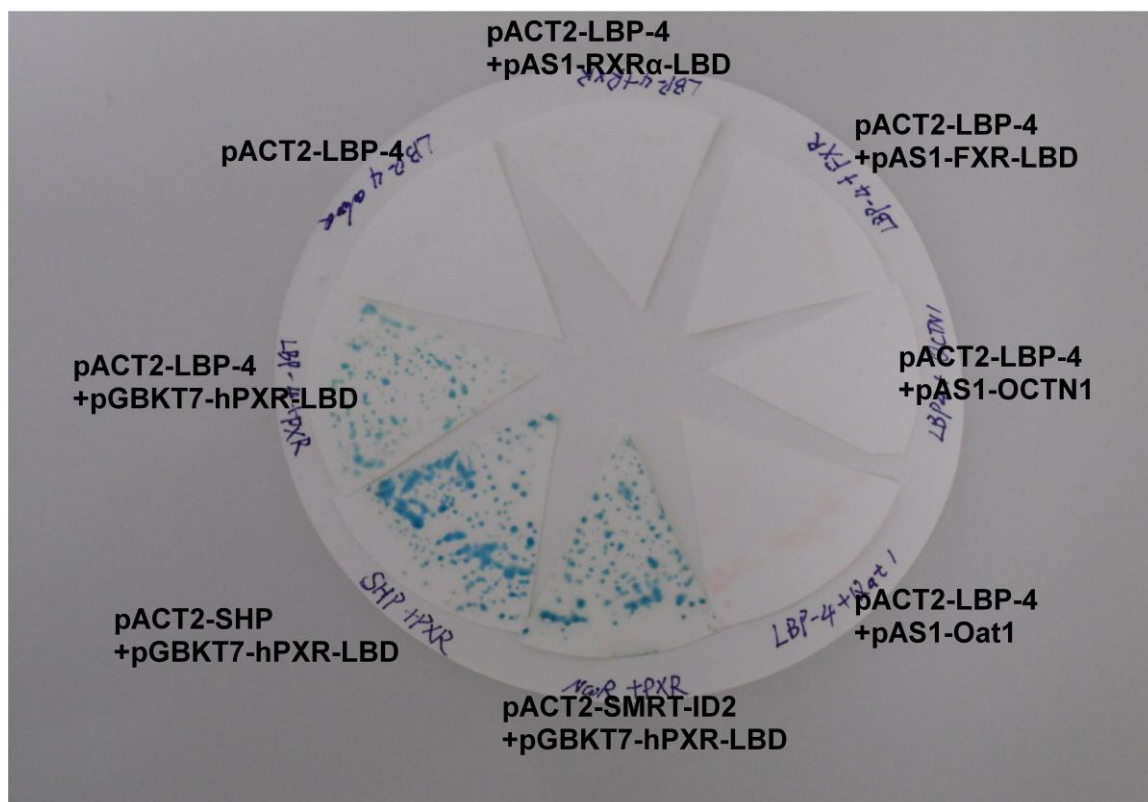
Figure 2-4.   Interaction between LBP-4 and Other Bait Proteins in Y153 Cells.   Y153 yeast cells harboring only prey plasmid (pACT2-LBP-4) were grew on Leu drop-out plates. Y153 yeast cells transformed with both prey (pACT2-LBP-4) and bait plasmids (as indicated in the figure) were grew on double drop-out (-Trp-Leu) plates.   The colonies were lifted and subjected to the X-gal test and the interaction was monitored through the appearance of the blue color.   SHP and SMRT-ID2 were employed as positive controls.

not other NRs.   To further confirm these results from Y153 cells, a different yeast strain,

Y190, was utilized.   In Y190 cells, LBP-4 also interacted with hPXR-LBD but not with

other testing proteins, consistent with what has been found in Y153 cells (Fig. 2-5).   Besides

changing yeast strains, another way to circumvent false positive interactions is to swap bait

and prey in the system (8).   In order to do that, LBP-4 was subcloned into pGBKT7 vector

and hPXR-LBD was subcloned into pACT2 vector.   After protein expression was verified

(data not shown), these two new constructs were co-transformed into Y190 cells and the blue

colonies formed after the colony-lift filter assay indicated that LBP-4 still interacted with

hPXR-LBD (Fig. 2-6).   While the X-gal test is only qualitative, we further employed another

quantitative method, quantitativeβ-galactosidase assay.   As shown in Fig.2-7, among all the

proteins tested, fold induction for the interaction between LBP-4 and hPXR-LBD was the

highest.

Confirmation of the Interaction Between hPXR-LBD and Novel PXR Interacting

Proteins in Mammalian Cells.   To overcome the differences between yeast and mammalian

cells, the mammalian two hybrid system was also utilized to confirm the interaction between

PXR interacting proteins and hPXR-LBD.   The principle underlying the mammalian two

hybrid system is quite similar to the yeast two hybrid system, but this system allows us to

detect the interaction in mammalian cells by utilizing a mammalian reporter gene (Fig.2-8A).

LBP-4, SUG1, SMRT-ID2 and SHP were subcloned into a pM vector from pACT2 vector to

generate the fusion protein of PXR interacting proteins and GAL4 DBD.   Unfortunately,

even though sequencing showed that the reading frame of pM-SHP was correct, the fusion

protein was not expressed in mammalian cells for some unknown reason.   As a result, we

Figure 2-5
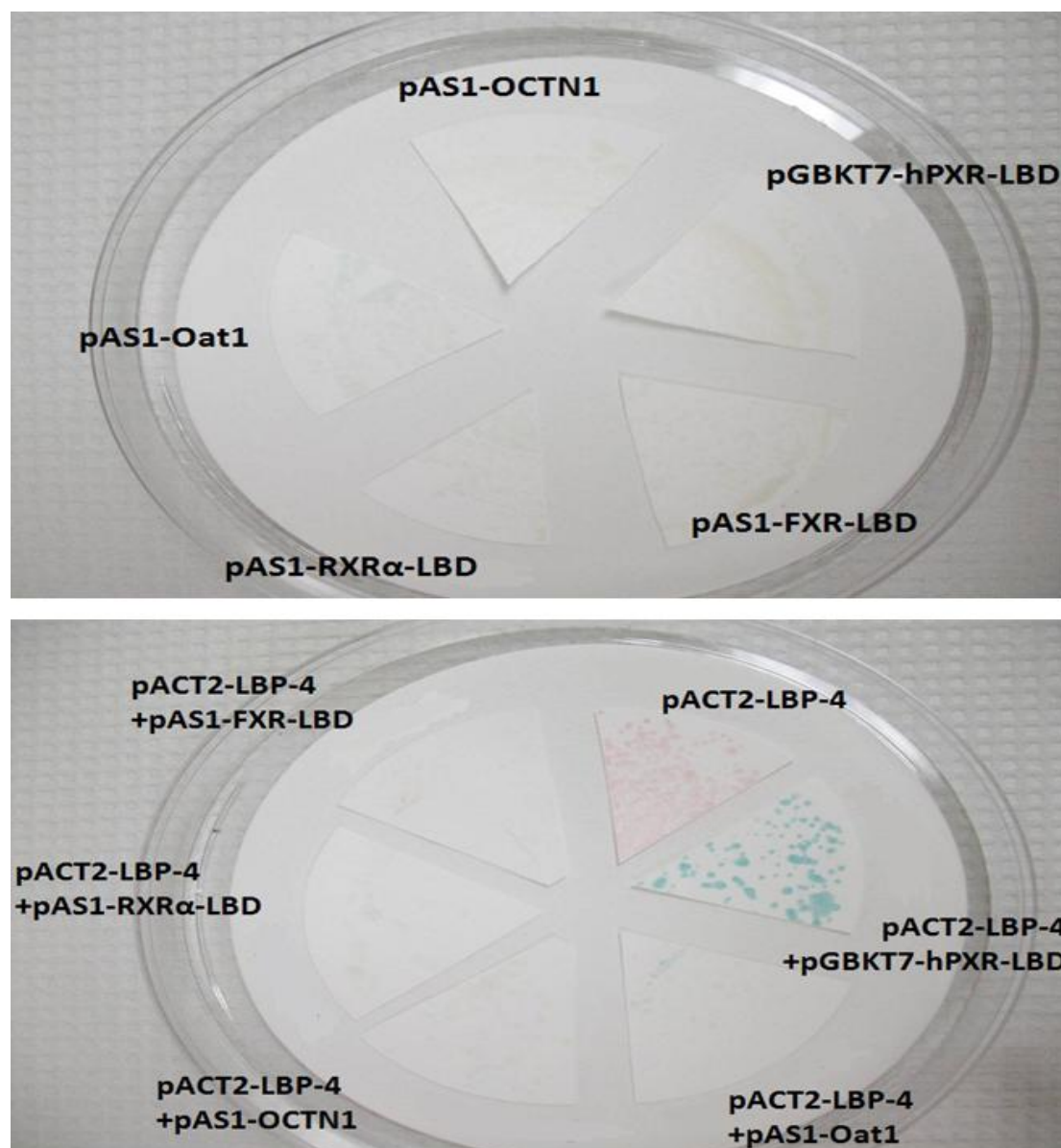
Figure 2-5. Interaction between LBP4 and hPXR-LBD in Y190 Cells. Y190 yeast cells harboring only bait plasmids (as indicated in the figure) were plated on Leu drop-out plates. Y190 yeast cells harboring both prey (pACT2-LBP-4) and bait plasmids (as indicated in the figures) were plated on double drop-out plates (-Trp-Leu). The colonies were lifted and subjected to the X-gal test. The interaction was monitored through the appearance of the blue color.

Figure 2-6
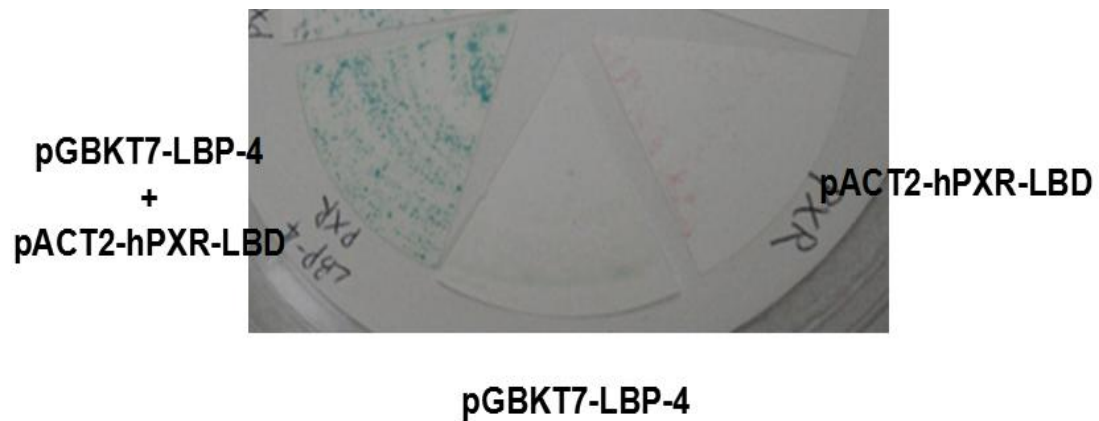
Figure 2-6.   Interaction between hPXR-LBD and LBP-4 in Y190 Cells After the Bait and Prey Were Swapped.   LBP-4 was subcloned into pGBKT7 vector and hPXR-LBD was subcloned into pACT2 vector.   The constructs were transformed into Y190 yeast cells as indicated in the figure.   The interaction between LBP-4 and hPXR-LBD was tested using the X-gal test.

Figure 2-7
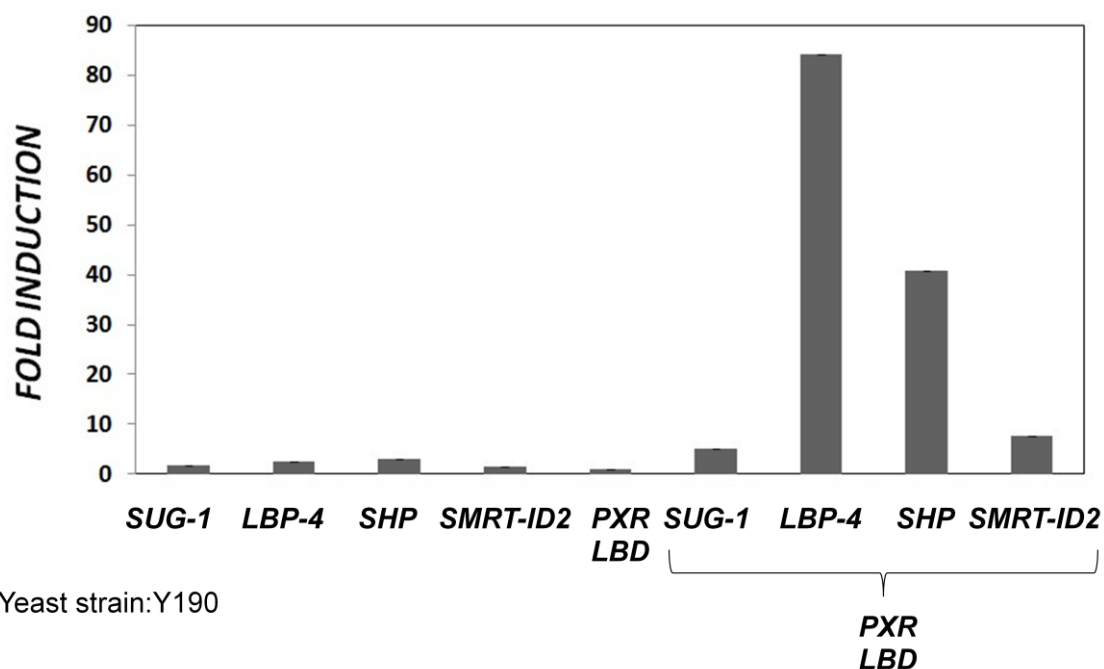
Figure 2-7. Quantitative Analysis of the Interactions between PXR and PXR Interacting Proteins. The strength of the interaction between PXR and cofactors was measured in the yeast two hybrid system. Different plasmids (as indicated in the figure) were transformed into the yeast strain Y190. Quantitative analysis of β-galactosidase activity was performed using a liquid β-galactosidase assay. All the cofactors are in pACT2 vector and PXR –hPXR is in pGBKT7 vector.

Figure 2-8

A
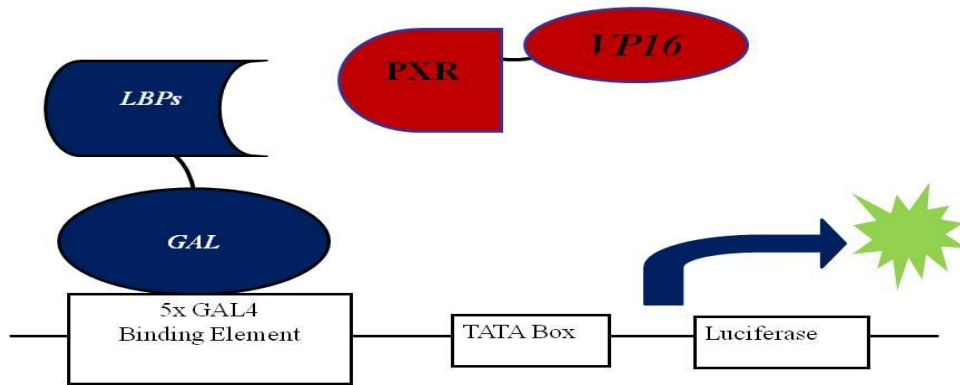
B
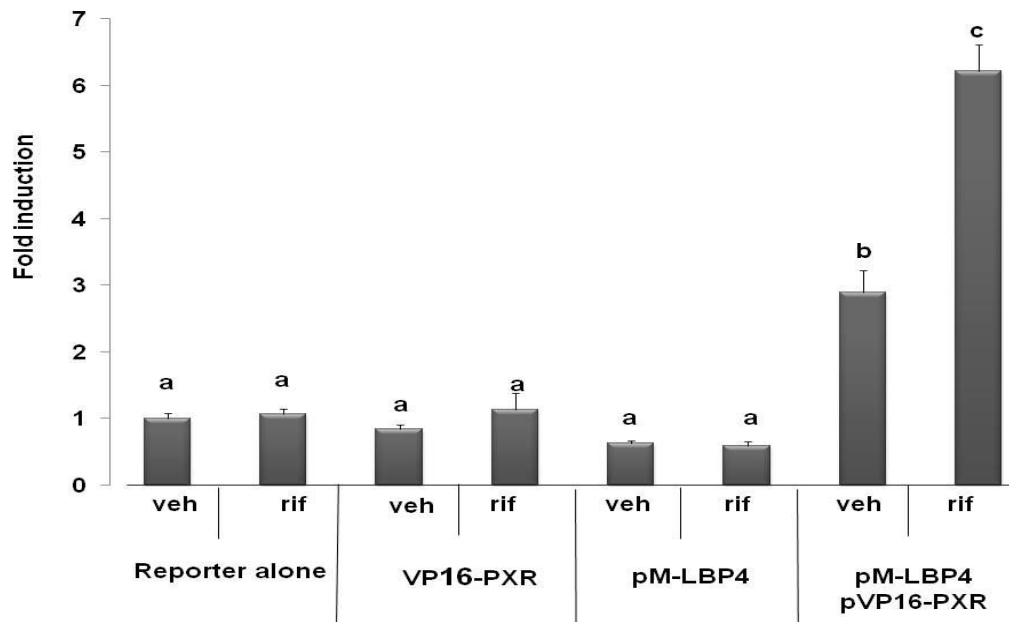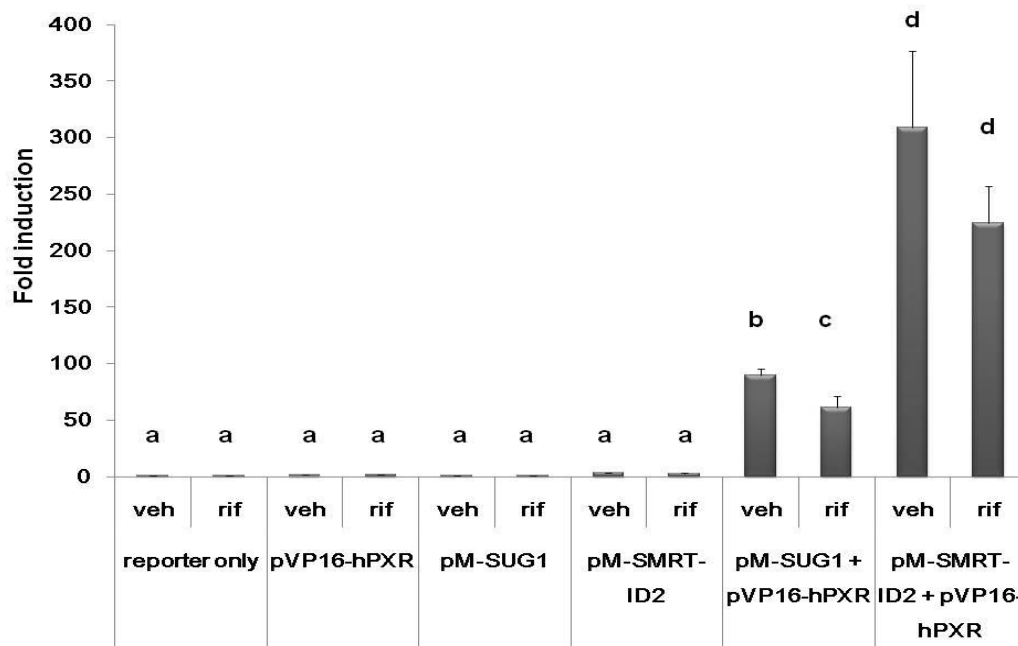
Figure 2-8. Interaction between hPXR-LBD and PXR Interacting Proteins in Mammalian Cells. (A) Schematic representation of mammalian two hybrid system. Cells were transfected with pFR-Luc, a 5xGAL4 binding element–luciferase reporter gene, and plasmids encoding VP16-PXR and GAL4 DBD fused to different LBPs. The interaction between LBPs and hPXR-LBD will lead to the expression of the reporter gene. (B) After transfection with indicated plasmids, cells were treated with 10 μM rifampicin for 24h before luciferase and β-galactosidase activities were measured. The transfection efficiency was normalized against the β-galactosidase activity from the cotransfected SV-β-gal. Results shown are fold induction over vehicle treated reporter only group and represent the averages and SEM from biquadratic assays.

could not further study the interaction between PXR and SHP in mammalian cells.   For the

other DBD-PXR interacting proteins, they were co-transfected with VP16-AD-PXR into

CV-1 cells.   Consistent with the data generated from yeast, LBP-4, SUG-1, SMRT-ID2 all

interacted with PXR in the CV-1 cells.   The interaction between PXR interacting proteins

and hPXR-LBD was affected by treatment with PXR ligand (Fig.2-8B).   For LBP-4, the

interaction increased after ligand treatment, while for SUG-1, the interaction decreased after

the same treatment.   The interaction between PXR and SMRT-ID2 slightly decreased after

rifampicin treatment, but the difference was not statistically significant.

2.4 Discussion

In this chapter, the yeast two hybrid system was used to explore new interacting proteins

for hPXR-LBD.   The identification of known PXR interacting proteins, SUG-1, SHP, and

SMRT, confirmed the validity of the screening.   From the screening, p0071 was also

identified as a potentially novel interacting protein for PXR.   The interaction between PXR

and the identified interacting proteins will be discussed below.

2.4.1 The Interaction between PXR and p0071.

p0071 is a member of the ARM repeat-containing protein family.   This protein family

is characterized by containing the tandem ARM repeats.   The members in this family can be

divided into three subfamilies referred to by representative members: β-catenin, p120, and

plakophilins (As shown in Fig. 2-9A).   LBP-4 is a fragment of p0071 containing the first 5

ARM repeats and part of the sixth ARM repeat of p0071 (Shown in Fig. 2-9B).

ARM repeat-containing proteins are well-known for their structural roles at anchoring

junctions, which is one type of cell-cell junctions.   There are two main categories of

anchoring junctions: adherens junctions and desmosomes.   These two anchoring junctions

share a similar structure and are distinguished primarily on the filament system to which they

attach.   In most cases, members of the β-catenin and p120 subfamilies assemble into

actin-associated adherens junctions, whereas the plakophilins assemble into intermediated

filament-based desmosomes.   In certain, conditions, some members, including plakoglobin,

p120, and p0071, can assemble into both adherens junctions and desmosomes, suggesting

their roles in regulating the cross-talk between the two kinds of junctions.

Besides their roles at cell-cell junctions, ARM repeat-containing proteins also undergo

nuclear translocation to play nuclear functions.   Through their dual presence, the ARM

repeat-containing proteins have the potential to allow changes in cell adhesion and junction

formation to affect transmembrane signaling and nuclear gene transcription.   Among the

family, β-catenin has been studied most thoroughly with respect to nuclear functions in the

canonical Wnt/β-catenin signaling pathway (9, 10).   This signaling pathway is regulated

through the controlled degradation of β-catenin in the cytoplasm.   In the absence of Wnt

ligands, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) and caseine

kinase 1 (CK1) within a degradation complex.   Phosphorylated β-catenin is then

ubiquitinated and targeted for degradation by the proteasome.   Through the quick

Figure 2-9

A

The Armadillo Protein Family
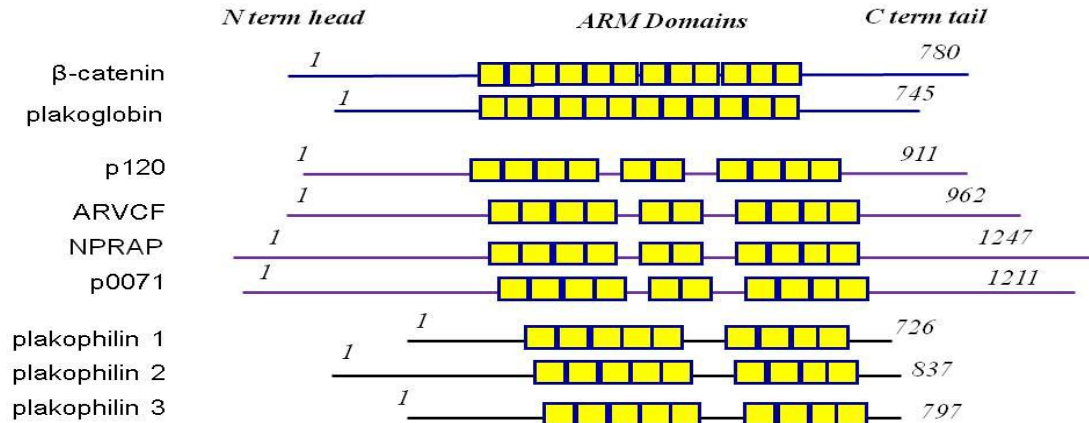
B

MPAPEQASLVEEGQPQTRQEAASTGPGMEPETTATTILASVKEQELQFQRLTRELEVERQIVASQLERCR

LGAESPSIASTSSTEKSFPWRSTDVPNTGVSKPRVSDAVHPNNYLIRTEPEQGTLYSPEQTSLHESEGSL

GNSRSSTQMNSYSDSGYQEAGSFHNSQNVSKADNRQQHSFIGSTNNHVVRNSRAEGQTLVQPSVANRAMR

RVSSVPSRAQSPSYVISTGVSPSRGSLRTSLGSGFGSPSVTDPRPLNPSAYSSTTLPAARAASPYSQRPA

SPTAIRRIGSVTSRQTSNPNGPTPQYQTTARVGSPLTLTDAQTRVASPSQGQVGSSSPKRSGMTAVPQHL

GPSLQRTVHDMEQFGQQQYDIYERMVPPRPDSLTGLRSSYASQHSQLGQDLRSAVSPDLHITPIYEGRTY

YSPVYRSPNHGTVELQGSQTALYRTGSVGIGNLQRTSSQRSTLTYQRNNYALNTTATYAEPYRPIQYRVQ

ECNYNRLQHAVPADDGTTRSPSIDSIQKDPREFAWRDPELPEVIHMLQHQFPSVQANAAAYLQHLCFGDN

KVKMEVCRLGGIKHLVDLLDHRVLEVQKNACGALRNLVFGKSTDENKIAMKNVGGIPALLRLLRKSIDAE

VRELVTGVLWNLSSCDAVKMTIIRDALSTLTNTVIVPHSGWNNSSFDDDHKIKFQTSLVLRNTTGCLRNL

SSAGEEARKQMRSCEGLVDSLLYVIHTCVNTSDYDSKTVENCVCTLRNLSYRLELEVPQARLLGLNELDD

LLGKESPSKDSEPSCWGKKKKKKKRTPQEDQWDGVGPIPGLSKSPKGVEMLWHPSVVKPYLTLLAESSNP

ATLEGSAGSLQNLSAGNWKFAAYIRAAVRKEKGLPILVELLRMDNDRVVSSVATALRNMALDVRNKELIG

KYAMRDLVNRLPGGNGPSVLSDETMAAICCALHEVTSKNMENAKALADSGGIEKLVNITKGRGDRSSLKV

VKAAAQVLNTLWQYRDLRSIYKKDGWNQNHFITPVSTLERDRFKSHPSLSTTNQQMSPIIQSVGSTSSSP

ALLGIRDPRSEYDRTQPPMQYYNSQGDATHKGPYPGSSKPSPIYISSYSSPAREQNRRLQHQQLYYSQDD

SNRKNFDAYRLYLQSSHSYEDPYFDDRVHFPASTDYSTQYGLKSTTNYVDFYSTKRPSYRAEQYPGSPDS

WV*

Figure 2-9. Members of the Armadillo Protein Family. (A) The members of armadillo protein family were illustrated. ARM repeat-containing proteins can be divided into three subfamilies referred to by representative members: 1. β-catenin (β-catenin, plakoglobin); 2. p120 [p120, NPRAP (neural plakophilin-related arm-protein), ARVCF (Armadillo repeat gene deleted in velo cardio-facial syndrome) and p0071]; and 3. plakophilins (plakophilin 1-3). The yellow boxes indicate ARM repeats. (B) Amino Acid sequence of p0071 and LBP-4. The amino acid sequence of p0071 was listed. The blue and pink boxes indicate the ARM repeats in p0071. The start and ending amino acid of LBP-4 was indicated with arrows.

degradation, the amount of β-catenin is maintained at low levels in the absence of Wnt ligands. Wnt ligands can bind to the cell surface receptor Frizzled and ultimately lead to inactivation of the degradation complex mentioned above. Inactivation allows the accumulation of β-catenin in the cytoplasm, which eventually leads to nuclear translocation of β-catenin. Upon entry into the nucleus, β-catenin interacts with transcription factors like T-cell factor (TCF) and lymphoid enhancer-binding factor (LEF) (11). β-catenin also interacts with transcription and chromatin co-regulatory proteins to regulate transcription (12, 13). Apart from TCF/LEF, β-catenin also interacts with many NRs and leads to divergent downstream outcomes (14-16).

Although the nuclear function of β-catenin is well-studied, the nuclear function of p0071 is still vague. When p0071 was first cloned, it was noticed that besides the presence at cell-cell borders, p0071 has a punctate cytoplasmic distribution pattern around the nucleus. A putative nuclear localization signal was also observed in p0071 (17).   In addition, p0071 is localized at the midbody during cytokinesis and is essential for Rho signaling during cell division (18).   While its function in the nucleus remains largely enigmatic, it is possible that p0071 has nuclear roles similar to other members in this family and this nuclear function might be displayed through its interaction with PXR.

It is known that β-catenin can mediate liver growth.   The loss of β-catenin can delay the liver regeneration after partial hepatectomy. Aberrant activation of β-catenin is often observed in hepatocellular malignancies (10).   Another important observation is that treatment of PCN, a potent PXR agonist, can result in increased liver mass in rats and mice and PXR is required for this PCN-induced hepatomegaly in mice   (19, 20).   As mentioned above, p0071 is localized at the midbody during cytokinesis and is essential for cell division (18).   It is plausible that p0071 may be involved in the regulation of liver growth through its

interaction with PXR.   Another interesting observation is that β-catenin can regulate the expression of cytochrome P450 (CYPs).   In hepatocyte-specific-β-catenin knockout mice, the expression of CYP1A2 and CYP2E1 is almost abolished in the liver, whereas CYP3A11, the expression of which is regulated by PXR, is unaffected (21).   While β-catenin is required for CYP1A2 and CYP2E1 expression, it is interesting to see whether p0071 regulates the expression of CYP3A11 and other PXR target genes.

2.4.2 The Interaction between PXR and SUG1.

Previous studies have shown that SUG1 interacts with mouse PXR in a progesterone-dependent manner (4, 22).   As one of the AAA (ATPases Associated with diverse cellular Activities) ATPases, SUG1 is one subunit of the 19S regulatory particle of the 26S proteasome (23).   The 26S proteasome is a huge protease complex with two major structural components: the 20S core particle and the 19S regulatory particle.   Structurally, the 20S core is cylindrical with a hollow cavity.   The 20S core harbors protease activities and is the place where proteins are degraded.   Two 19S regulatory particles are located at each end of 20S to control the entry to the 20S core.   The 19S regulatory particles recognize targeted substrates, unfold them, cleave the attached ubiquitin chains, open the 20S core and drive the unfolded polypeptide into the cavity of 20S for degradation.

Besides its structural role in the proteasome, accumulating evidence indicates that SUG1 is closely associated with transcription in both proteolytic and non-proteolytic ways.   In fact, before its identification as a subunit of the proteasome, SUG1 was reported to be a component of the RNA polymerase II holoenzyme in yeast (24).   Later work in yeast further confirms that SUG1 is required for efficient elongation of RNA polymerase II and also contributes to the regulation of histone modification (25, 26).   Mammalian SUG1 is also closely related to transcription.   Mouse SUG1 has been shown to interact with the AF-2 domain of many NRs

in yeast two hybrid system (27).   In mammalian cells, overexpression of SUG1 suppresses both the constitutive and ligand-mediated activity of hCAR1 (28).   SUG1 also regulates the expression of RARα target genes.   Ferry *et al.* showed that both overexpression and knockdown of SUG-1 led to reduced expression of RARα target genes.   Using CHIP and Re-CHIP, they demonstrated that through the interaction with SRC-3, SUG1 was recruited to the promoters of RARα target genes and mediated the proteasomal degradation of SRC-3. While the knockdown of SUG1 inhibits the degradation of SRC-3 upon ligand treatment, ligand-induced degradation of RARα is not affected, suggesting substrate specificity for degradation.   This study further proves that SUG1 links transcription and degradation processes (29).

In our yeast and mammalian two hybrid systems, SUG1 interacts with hPXR-LBD. Previously, it has been shown that both progesterone and endocrine-disrupting chemicals like phthalic acid and nonylphenol can activate PXR.   Overexpression of SUG1 inhibits progesterone-mediated transcription of PXR but not endocrine-disrupting chemicals-mediated transcription (22).   While it is clear that the effects of SUG-1 on PXR transactivation are differential with various PXR agonists, the underlying mechanism(s) requires further investigation.   Our understanding of the proteasome and its role in regulating transcription is increasing rapidly, but the exact role of SUG1 is still unclear.   Whether SUG1 facilitates the assembly of transcription complex, functions as a chaperone to selectively facilitate the degradation of certain proteins, or simply recruits the proteasome to the promoter of target genes requires further investigation.   Future studies will be required to characterize the SUG1-associated complexes and how they participate in transcription in both proteolytic and

non-proteolytic ways.

2.4.3 The Interaction between PXR and SHP.

The lack of the DNA binding domain makes SHP an atypical member of the NR family. Highly expressed in the liver, SHP plays an important role in regulating bile acid homeostasis and its expression is under the control of another liver-enriched NR- FXR (30, 31). Bile acids are synthesized in hepatocytes through a multi-step process. The first and rate limiting step is catalyzed by cholesterol 7-α-hydroxylase, also known as cytochrome P450 7A1 (CYP7A1). SHP has been shown to negatively regulate CYP7A1 gene expression through its interaction with LRH-1, a positive regulator of CYP7A1 expression (31, 32). When bile acid levels increase, they can activate FXR to induce the expression of SHP. Through the inhibition of CYP7A1, SHP reduces the production of bile acids, forming a feedback loop to maintain bile acid homeostasis.

Besides LRH-1, SHP has been shown to interact with other NRs (30, 33-37). The interaction between SHP and NRs often leads to decreased transcriptional activity of these NRs, because the binding between SHP and NRs alters the interaction between NRs and co-regulatory proteins. Though, there are some exceptions: SHP has been shown to augment the transcriptional activities of PPARα and PPARγ (38, 39).

SHP can interact with PXR in a ligand dependent manner to suppress the transcriptional activity of both human and mouse PXR. The interaction between PXR and SHP inhibits the binding of SRC1 to PXR and also decreases the DNA binding ability of PXR (5). In our

yeast two hybrid assay, we identified SHP as an interacting protein for hPXR-LBD. Increasing evidence suggests that the regulation between SHP and PXR occurs at multiple levels.   While SHP inhibits PXR transcriptional activity, the activation of PXR also leads to decreased SHP expression (40).   Moreover, just like SHP, the expression of PXR is also regulated by FXR, indicating the intense cross talk between different NRs (41).

2.4.4 The Interaction between PXR and SMRT

SMRT is a well-known transcriptional regulatory protein that suppresses the transcriptional activity of many transcription factors.   The SMRT that we identified from the human liver cDNA library is a C-terminal fragment.   The protein encoded by the cDNA contains the well-identified NR-interacting domain-2 (ID-2) but not ID-1 of SMRT, which is in accordance with the previous finding that hPXR preferably binds to ID2 of SMRT (42).

It is proposed that without ligands PXR is associated with co-repressors like SMRT to actively inhibit target gene expression.   The binding of ligand leads to a conformational change of PXR that dissociates corepressors and allows the recruitment of coactivators and basal transcriptional machinery to initiate transcription (6).   However, in our mammalian two hybrid assay, rifampicin treatment did not significantly decrease the interaction between PXR and SMRT.   At first glance, our results contradict the NR activation model.   However, other research groups have also observed an interaction between PXR and SMRT in the presence of rifampicin (43, 44).   Two major splicing isoforms have been identified for SMRT: α and τ, where SMRTα contains an extra 47-amino acid sequence after the ID2 motif (45).   These two isoforms had similar cellular distribution patterns and similar transcription

repression activities. However, they interact with PXR in different patterns. Whereas the interaction between SMRTτ and PXR can be dissociated by ligand treatment, the interaction between SMRTα and PXR is resistant to ligand. The SMRT in our assay is the fragment of SMRTα, and our results that rifampicin did not significantly decrease the interaction between PXR and SMRT are consistent with the findings from Li and colleagues (46).

The two different IDs in SMRT exhibit different binding affinities toward different NRs. Different NRs also have different preferences toward the two SMRT isoforms. Moreover, the expression patterns of the two isoforms are diverse in different tissues, which might contribute to the tissue-selective effects of NRs (45). Since the interaction between PXR and SMRTτ can be dissociated by ligand treatment, it is plausible to assume that the PXR-SMRTτ interaction may be more relevant in the inductive response of PXR activation by ligands, while the PXR-SMRTα interaction may be more related to PXR ligand induced repression.

2.5 Conclusions

It is clear that signaling pathways can regulate PXR function. Some xenobiotics can indirectly activate PXR by modulating cellular signaling pathways. The identification of novel interacting proteins for PXR is an important field which will undoubtedly improve our understanding of PXR activities and the signaling pathways that regulate its function.

The yeast two hybrid system is easy to perform, the sensitivity of the assay is high, and since it is performed in a higher eukaryotic system, it can overcome the deficiencies in bacterial cells or in *in vitro* systems where some post-translational modifications might be unavailable. Because of the advantages mentioned above, the yeast two hybrid system has been widely used to detect known proteins for interactions, to define domains for interactions, and to screen cDNA libraries for novel interaction proteins. Inspired by the yeast two hybrid system, other systems have been developed to study biomolecular interactions, such as the mammalian two hybrid system that detects protein-protein interactions, the one hybrid system that detects DNA-protein interactions (47, 48), and the three hybrid system that detects RNA protein interactions (49). While the yeast two hybrid system is a good method to screen different cDNA libraries for novel interacting proteins, the difference between yeast and mammalian cells needs to be considered. It is necessary to confirm the results from yeast in mammalian cells. Nonetheless, the yeast two hybrid system is an excellent method for initial screening purposes.

From our screening results, both novel and known proteins that interact with hPXR-LBD were identified. As an important xenobiotic sensor which also plays important roles in many physiological and pathological processes, the full understanding of PXR function and related signaling pathways will definitely benefit the clinic. Sixteen years after the identification of PXR, the regulation of PXR activity is still under active investigation. Through the finding of new interacting proteins and the refinement of the currently recognized roles, the discovery of new, meaningful functions for PXR can be anticipated. The new understanding of PXR function will lead to the development and identification of promising novel drugs.

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2.6 References

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Chapter 3: Characterization of PXR Ubiquitination

3.1 Introduction to Ubiquitin Proteasome System (UPS)

In the yeast two hybrid screening described in chapter 2, SUG1 was identified as a PXR-interacting protein. As one of the AAA (ATPases Associated with diverse cellular Activities) proteins, SUG1 is a subunit of the 19S regulatory particle of the 26S proteasome (1). The mammalian 26S proteasome is a large protease complex with two major structural components: the 20S core particle and the 19S regulatory particle. The 20S core harbors protease activities and is where proteins are degraded. Two 19S regulatory particles are located at each end of the 20S to control the entry of proteins into the 20S core. The 19S regulatory particle recognizes targeted substrates, unfolds them, cleaves off the attached ubiquitin chains, opens the 20S core and drives the unfolded polypeptides into the cavity of the 20S core for degradation.

To maintain the cellular homeostasis, the 19S regulatory particles and the 20S core particle form the 26S proteasome to eliminate misfolded or damaged proteins, unassembled polypeptide chains, short-lived regulatory proteins and abnormal proteins. However, this process must be strictly regulated, so that abnormal proteins can be eliminated efficiently while functioned proteins are not degraded unintentionally. This regulation is achieved through regulated ubiquitination, which labels unwanted proteins for degradation. In most cases, proteins are selected for proteasomal degradation based on the presence or absence of ubiquitination. However, exceptions do occur where some proteins are degraded by the

proteasome without being previously ubiquitinated (2, 3).

Ubiquitination is a type of post-translational modification that occurs when ubiquitin, a small regulatory protein, is covalently attached to the lysine residue(s) of target proteins. This process requires the function of a cascade of enzymes, including E1 (ubiquitin-activating enzyme), E2 (ubiquitin-conjugating enzymes) and E3 (ubiquitin ligases). During the ubiquitination process, ubiquitin is initially activated by E1 in an ATP-dependent manner, which leads to the attachment of ubiquitin to the E1 enzyme through a thioester bond. Activated ubiquitin is then transferred to E2 through a trans(thio)esterification reaction. E3 facilitates the final step of ubiquitination where the carboxyl terminus of ubiquitin is attached to the ε-amino group of a lysine in target proteins through an isopeptide bond (4). A normal cell typically contains a few E1s and dozens of E2s. While the number of E1 and E2 enzymes is limited, hundreds of E3s have been identified. It is believed that substrate recognition is achieved through different types of E3s (5, 6).

Mono-ubiquitination, multi-mono-ubiquitination and poly-ubiquitin chains can be formed through attachment to lysine residue(s) within substrates and the lysine residue(s) within ubiquitin. There are seven lysines in ubiquitin, K6, K11, K27, K29, K33, K48 and K63, all of which can be involved in ubiquitin chain formation. Moreover, the ubiquitin chain can also be formed via the N-terminal methionine of each ubiquitin (7). The linkage type of the ubiquitin chain determines the fate of ubiquitinated proteins, since different linkages give rise to distinct three-dimensional topologies that can lead to specific recognition with various interacting proteins (8). Poly-ubiquitination through K48 of each ubiquitin usually labels modified proteins for degradation by the 26S proteasome (9). Poly-ubiquitin

chains linked through other lysines can lead to nonproteolytic outcomes, such as activation of protein kinases, activation of transcription factors, and the orchestration of DNA repair processes. In addition to the K48-linked poly-ubiquitin chain, poly-ubiquitin chain formed on K63 of ubiquitin has also been intensively-studied. K63-linked poly-ubiquitin chain usually mediates the recruitment of binding partners for ubiquitinated substrates and plays important roles in DNA repair and protein kinase activation in the NF-κB pathway (10).

Ubiquitin and poly-ubiquitin chains attached to substrates can be disassembled by deubiquitination enzymes. Therefore, ubiquitination is a dynamic and reversible process, which also makes it well-suited to regulate dynamic processes like transcription.

3.2 Regulation of Gene Expression by UPS

The UPS is closely related to transcription. The proteasome and its subunits have been proposed to regulate transcription in both proteolytic and non-proteolytic ways. Transcription can be divided into three distinct steps: transcription initiation, elongation, and termination. The proteasome and its subunits play pivotal roles in all steps and control both the magnitude and temporal aspects of gene expression.

3.2.1 Proteolytic Roles of UPS in Transcription

Transcription is a dynamic process and the binding of transcriptional factors to target gene promoters is essential for initiating transcription. On the other hand, timely removal of transcription factors is of equal importance for efficient transcription. Proteasomal degradation is required for the dynamic exchange of transcription factors, co-regulatory proteins, and basal transcriptional machinery to ensure that the correct proteins are present at

the right place and time for transcription to proceed efficiently.

It is interesting to note that for most unstable transcription factors, their transcription activation domains overlap with sequences that are responsible for UPS-dependent degradation (degrons), revealing a convergence of these two different processes (11). In both yeast and mammalian cells, transcriptional activity can be impaired when proteasomal degradation is inhibited, indicating its importance in regulating transcription. In yeast cells, transcription activation by Gcn4, Gal4 and Ino2/4 are all inhibited by MG132 (N-benzyloxycarbonyl-L-leucyl-L-leucyl-L-leucinal), which is a potent and specific proteasome inhibitor (12). In mammalian cells, ERα has been shown to be degraded by the proteasome. Blocking proteolysis of the proteasome using MG132 attenuates ligand-dependent transactivation despite the fact that the protein levels of ERα are increased (13). Through the study of ERα target gene pS2 in MCF-7 cells, Metivier *et al.* showed that ERα and the transcriptional machinery were recruited to the pS2 promoter in a cyclical manner and each cycle corresponded to a single round of transcription (14). Without proteasomal degradation, the promoter is occupied and a new round of transcription cannot be initiated. Similar to ERα, inhibiting proteasomal degradation also suppresses AR-, PR- and CAR-mediated expression of their target genes. MG132 prevents the release of AR from the promoter of prostate-specific antigen (PSA) and blocks androgen-induced PSA mRNA accumulation (15). Inhibition of the 26S proteasome blocks PR-dependent transcription due to failed recruitment of RNA polymerase II (16). In HepG2 cells, MG132 treatment causes CAR to accumulate in the cytoplasm and attenuates TCPOBOP-induced CAR transcriptional activation on reporter constructs containing CAR-binding DNA elements derived from human

CYP2B6 gene (17). Similar results are also obtained from primary cultures of human hepatocytes, where proteasomal inhibition represses CAR nuclear trafficking, disrupts the interaction between CAR and nuclear coactivators, and inhibits the expression of target genes following ligand treatment (18). However, in contrast to the NRs mentioned above, GR-mediated gene activation is enhanced by blocking proteasomal activities (19, 20). Inhibiting proteolysis of the proteasome has been shown to block GR mobility, immobilize GR within the nuclear matrix, and increase its transcriptional output.

The differential influences of proteasomal inhibition on the function of different NRs suggest a sophisticated relationship between the proteasome and the NR family. From the results discussed above, it is plausible to propose that the 26S proteasome might differentially regulate the activities of different NRs. However, in the examples cited above, only a few target genes of each NR are examined in the study. Using a genomic approach, Kinyamu *et al.* examined the impact of proteasomal inhibition on GR- and ER-mediated gene transcription in MCF-7 cells. The results indicate that inhibition of proteasome activity affects gene expression by GR and ER in a similar manner. For both GR and ER, after proteasomal inhibition, some target genes are upregulated, whereas some are downregulated, suggesting these two receptors are similar in their requirement of proteolytic activity for target gene expression. In addition, this also suggests that the requirement for proteolytic activity is gene specific, instead of NR specific (21).

Besides promoter clearance, the 26S proteasome is also associated with transcriptional termination. The 26S proteasome has been shown to be located at regions that correlate with

RNA polymerase II build-up, such the 3′ ends of genes, sites of UV damage and other regions that present transcriptional pause sites. The activities of proteasomal degradation are proposed to resolve these stalled complexes. While inhibition of the 19S decreases transcriptional elongation, inhibition of the 20S has been shown to decrease transcriptional termination (22). Furthermore, the proteasome can regulate transcription on other levels. In the canonical NF-κB1 signaling pathway, NF-κB1 is first synthesized as a 105 kDa precursor, which needs to be processed by ubiquitination and proteasome-dependent trimming to become the mature p50 transcription factor (23). After maturation, p50 is held in the cytoplasm by its inhibitor protein IκBα. When stresses are presented to activate the NF-κB pathway, IκBα is phosphorylated, ubiquitinated, and then degraded by the 26S proteasome. Degradation of IκBα allows NF-κB to translocate into the nucleus and activate its target genes. Thus, the UPS functions not only in the regulated processing of precursors into active proteins, but also in the degradation of inhibitor protein of transcription factors (23).

3.2.2 Non-Proteolytic Roles of UPS in Transcription

SUG1 was previously recognized as a transcriptional regulator before its identification as a component of the 19S regulatory particle of the 26S proteasome (24). Subsequently, a second yeast 19S subunit, Sug2/Rpt4, is also found to interact with the yeast transcriptional activator Gal4 (25). At first, these findings were suggested to link proteolytic events with transcription. However, mutations that block the proteolytic functions of the 20S subunit do not affect the roles of SUG1/2, suggesting that proteolysis is not required and that SUG1/2 might have non-proteolytic functions (26). Another piece of evidence suggesting

non-proteolytic functions of the proteasome is from genome-wide chromatin immunoprecipitation (ChIP) analysis of yeast. The results revealed that proteasomal proteins bind to the majority of yeast genes. There is widespread overlap between 19S and 20S subunit binding sites, suggesting that these subunits might constitute the 26S proteasome at those sites. However, there are some genes which are only associated with either the 19S or the 20S subunit, but not both, indicating these subunits may function independently of one another and that the intact 26S proteasome proteolytic function is not required (27, 28). 19S ATPases are localized both at promoters and transcribed regions, and are suggested to regulate transcriptional initiation and elongation (29, 30). The 20S associates mainly with the 3′ ends of genes and is suggested to contribute to transcriptional termination (22).

19S ATPase activity has also been shown to be essential for the formation of transcription pre-initiation complexes (PIC) (31). Both SAGA (Spt-Ada-Gcn5-acetyltransferase) and TFIID (Transcription factor-IID)-dependent transcriptional activation have been characterized in yeast cells (32). SAGA is a multi-subunit complex with histone acetyltransferase (HAT) and deubiquitinase activities. TFIID is composed of TBP (TATA-box binding protein) and 14 different TBP-associated factors (TAFs). SAGA and TFIID interact with transcription activators to stimulate transcriptional initiation and activation. Both SAGA and TFIID complexes are highly conserved from yeast to human, and play crucial roles in eukaryotic gene activation (32). Lee *et al.* reported that the 19S regulatory particle enhanced the interaction between Gal4 and SAGA. This enhancement is dependent on 19S ATPase activity, but not the 20S core particle (33). Similarly, regulatory roles of the 19S regulatory particle at TFIID-dependent

genes have also been observed. The 19S regulatory particle but not the 20S core particle is recruited to RPS5 gene promoter to enhance the recruitment of TFIID to the promoter for transcriptional initiation, extending this non-proteolytic function of the proteasome in transcription (34). The 19S regulatory particle has also been shown to enhance elongation independent of proteolysis. Transcriptional elongation is defective upon inactivation of Sug1. In contrast, inactivating proteolytic function of the 20S core particle does not affect elongation (30). The same effect is also confirmed in mammalian systems, indicating that the nonproteolytic activity of the 19S regulatory particle maybe a common mechanism in transcription in eukaryotes (35).

Besides the proteasome, ubiquitination modification also directly affects transcriptional activities of transcription factors, such as in the case of p53. p53 is a key regulator of cell-cycle control, apoptosis, and genomic stability. Studies indicate that ubiquitinated p53 is bound to promoters of genes associated with growth arrest but not genes associated with apoptosis, suggesting that p53 ubiquitination contributes to its selection of transcriptional targets (36). Another example is the yeast transcription activator, Met4, which regulates sulfur metabolism in yeast. Ubiquitylation of Met4 turns off the expression of its target genes, without invoking its proteolysis (37).

As a ligand activated-NR, PXR is an important regulator of xenobiotic metabolism and clearance. Studies indicate that PTMs like phosphorylation also affect PXR activities, representing another level of regulation (38). However, the ubiquitination of PXR has not been fully studied. Previous studies demonstrate that mouse PXR interacts with SUG1, and

a potential E3 ligase for PXR has been identified (39-41). In our laboratory, human PXR ligand binding domain was found to interact with SUG1 in a yeast two hybrid screening and this interaction was further confirmed in mammalian cells. Here we identify that PXR is a target of UPS and that the UPS affects the transactivation capacity of PXR. Moreover, the crosstalk between ubiquitination and SUMOylation of PXR is explored. These data provide an additional link between nuclear receptor-mediated gene transcription and the UPS.

3.3 Materials and Methods

Plasmids and Chemicals. Unless otherwise indicated, all chemical compounds were purchased from Sigma and all cell culture supplements were purchased from Gibco. The full-length mouse and human PXR expression vectors were described previously (42, 43). To construct the FLAG-tagged mouse PXR expression vector, the cDNA encoding murine PXR was excised from pSG5-mPXR expression vector using EcoRI and inserted into pCMV-Tag 2B (Agilent). To construct the FLAG-tagged human PXR expression vector, the cDNA encoding human PXR was excised from pSG5-hPXR expression vector using EcoRI and SalI sites and was inserted into pCMV-Tag 2B (Agilent) using EcoRI and SalI sites. pRK5-HA-Ubiquitin-WT, K48R and K63R plasmids were obtained from Addgene. HA-ubiquitin-WT, K48R and K63R were subcloned into pcDNA4/Hismax A expression vector (Invitrogen) using EcoRI and NotI sites. pcDNA4/Hismax-HA-Ubiquitin-K48, 63R was generated using the QuikChange Mutagenesis system (Stratagene). pcDNA4-Hismax-Ubiquitin-K48R was used as template and the primer used for site-directed mutagenesis are as follows:

5'-GCTGTCTGATTACAACATTCAG*AGG*GAGTCCACCCT-3'

5'-AGGGTGGACTC*CCT*CTGAATGTTGTAATCAGACAGC-3'

Non-His-tagged SRα-HA-SUMO1 and pcDNA3-HA-SUMO3 plasmids were obtained from Addgene.

Cell-based Ubiquitination Assay.   Hepa1-6 cells were transfected using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions.   48 hours after transfection, the cells were harvested in Gua 8 buffer (6M guanidinium-HCl, 10mM Tris, 100mM sodium phosphate buffer, pH 8.0), sonicated, the cell lysates cleared by centrifugation and mixed with 30μl of Talon metal affinity resins (Clontech) equilibrated in Gua8 buffer.   The mixture was incubated with rotation for 2 hours at room temperature and washed twice in Gua 8 buffer, three times in Urea 6.5 buffer [8M urea (Fisher Scientific), 10mM Tris, and 100mM sodium phosphate buffer, pH 6.5, supplemented with 20mM imidazole (Fisher Scientific)] and once in 1×PBS.   After the final wash, the beads were resuspended in 30μl of 2×SDS-PAGE loading buffer supplemented with 50mM DTT (dithiothreitol), boiled for 5 min, and the proteins resolved by 10% SDS-PAGE.   The gel was transferred to polyvinylidene difluoride membrane and probed with anti-PXR antibody (sc-48340, Santa Cruz) or anti-HA antibody (MMS-101P, Covance).

Primary Human Hepatocyte Culture, Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis.   The primary human hepatocytes were derived from samples collected and provided by the University of Kansas Medical Center, Department of Pharmacology, Toxicology and Therapeutics Hepatocyte Core Lab and the KU Liver Center.   Freshly isolated human hepatocytes were plated at a density of 5×10^5 cells/well in 12-well plates coated with 0.2 mg/ml type I collagen.   Isolated

hepatocytes were maintained in Dulbecco's Modified Eagle's Medium supplemented with 100 nM dexamethasone, 100 nM insulin, 100U/ml penicillin G, 100µg/ml streptomycin, and 5% bovine calf serum and kept at 37 ℃ in a humidified incubator with 95% air and 5% CO_2. Hepatocytes were allowed to attach to the plate for 4 hours and then the medium were changed to William's Medium E supplemented with 100 nM dexamethasone, 0.1 mg/ml Matrigel (BD Bioscience), 2 mM L-Glutamine, 1×ITS, 100U/ml penicillin G, and 100µg/ml streptomycin. Cells were treated with vehicle (DMSO), 10 µM rifampicin, or 10 µM MG132 for the indicated time points. Total RNA was isolated from hepatocytes using the RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. 2µg of RNA was reverse transcribed using random primers (Promega). Reverse transcription cycling conditions were 25 ℃ for 10 min, 42 ℃ for 60 min, and 95 ℃ for 5 min. Equal amounts of cDNA were used in real-time quantitative polymerase chain reactions (RT-qPCRs). Reactions included 1 X Power SYBR Green (Applied Biosciences) and 300nM gene-specific primers. The sequences for primers are listed below:

Genes	Primers
β-actin	5'-CAAGATCATTGCTCCTCCTG-3'
	5'-TCATAGTCCGCCTAGAAGCA-3'
CYP3A4	5'-CAGGAGGAAATTGATGCAGTTTT-3'
	5'-GTCAAGATACTCCATCTGTAGCACAGT-3'

RT-qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems).   Cycling conditions were 95 ℃ for 10 min followed by 45 cycles of 95 ℃ for 10s and 60°C for 1 min.   All data were normalized to β-actin and fold induction was calculated using the $\Delta\Delta^{ct}$ method.

3.4 Results

Ubiquitination of PXR in Hepa1-6 Cells.   Previously, our laboratory has shown that PXR is ubiquitinated in HeLa cells.   In the present study, Hepa1-6 cells were used to assess the ubiquitination level of PXR.   Hepa1-6 is a mouse hepatoma cell line that harbors liver specific properties (44).   Since PXR is mainly expressed in the liver and intestine, Hepa1-6 cells are more relevant to our study compared to HeLa cells.   Utilizing the previously described mammalian ubiquitin expression vector in which an N-terminal HA-epitope was fused to ubiquitin, a novel ubiquitin expression vector containing an extended N-terminus to include a $(His)_6$-tag and an X-press tag followed by an HA epitope fused in frame to ubiquitin was generated.   The resulting form of ubiquitin adds approximately 17 kDa to the size of PXR in our cell-based ubiquitination assay.   In the assay, $(His)_6$-ubiquitin and PXR were co-transfected into Hepa1-6 cells.   Affinity resin was used to enrich $(His)_6$-tagged ubiquitin and ubiquitinated proteins.   As shown in Fig. 3-1, when PXR and $(His)_6$-ubiquitin were co-expressed in cells, a single prominent band was observed above the unmodified PXR band, indicating that PXR was mono-ubiquitinated.   After treatment of MG132, high molecular
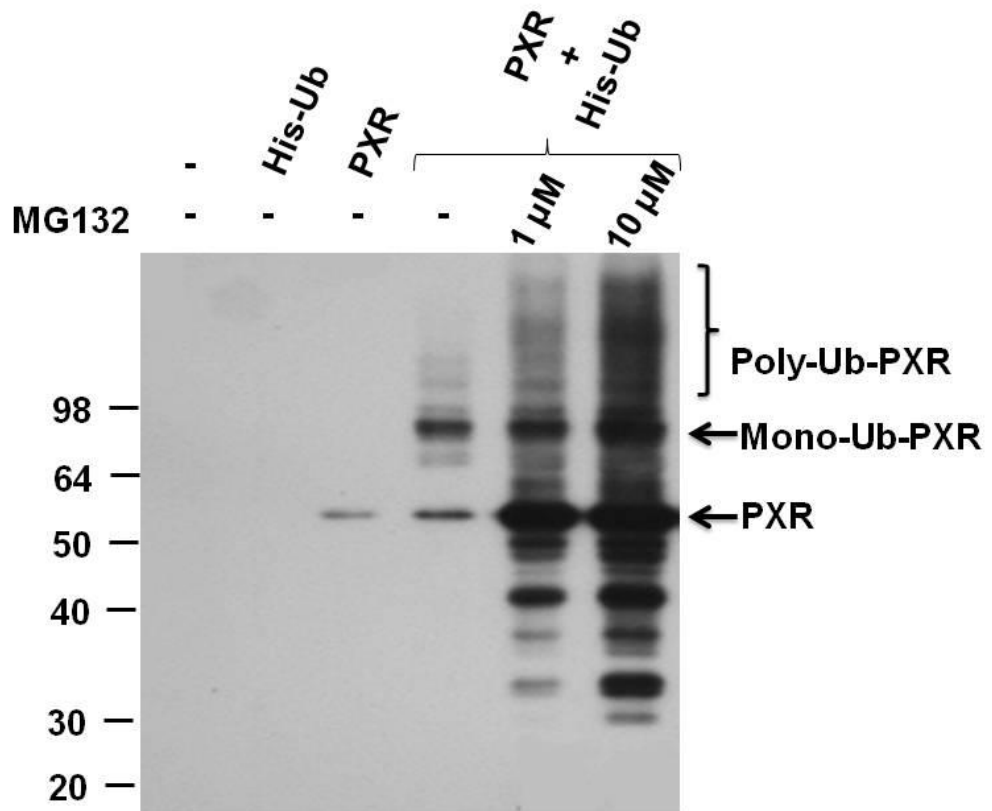
Figure 3-1

Figure 3-1. Detection of Ubiquitinated PXR in Hepa1-6 Cells. Hepa1-6 cells were transfected with indicated plasmids to express PXR and/or His-tagged wild type ubiquitin. Cells were treated with MG132 for 18 hours, and then lysed in denaturing buffer. Ubiquitinated proteins were purified by using cobalt-linked agarose beads. The blot was probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein (sc-48340, Santa Cruz).

weight smears began to appear, suggesting the formation of ubiquitin chains.   Moreover, the chain formation was enhanced upon increasing MG132 concentration.   These data suggest that in Hepa1-6 cells, PXR is a molecular target of the ubiquitin-proteasome pathway.

The Characterization of the Linkage Type of Ubiquitin Chains on PXR.   While ubiquitination of PXR has previously been demonstrated, the precise molecular nature of the ubiquitin linkage and its influence on the biological activity of PXR is unknown.   Expression vectors for $(His)_6$-tagged wild type ubiquitin, K48R ubiquitin (lysine 48 in ubiquitin is mutated to arginine), K63R ubiquitin (lysine 63 in ubiquitin is mutated to arginine), and K48, 63R ubiquitin (lysines 48 and 63 in ubiquitin are mutated to arginines) were generated. Hepa1-6 cells were co-transfected with expression vectors encoding ubiquitins and PXR and then subjected to the cell-based ubiquitination assay.   As shown in Fig. 3-2, heavily ubiquitinated PXR was detected when PXR and wild type ubiquitin were co-expressed in the cells.   When Ub-K48R was co-expressed with PXR, ubiquitin chain formation was reduced. However, when Ub-K63R was co-expressed with PXR, ubiquitin chain formation was completely intact compared to wild type ubiquitin.   When the double mutant Ub-K48, 63R construct was used in the assay, ubiquitin chain formation was also decreased, while the appearance of a single prominent band indicated that PXR was still mono-ubiquitinated. Taken together, these data indicate that K48 is the primary site of chain formation on ubiquitin that is linked to a single lysine residue in PXR protein.   It is widely recognized that ubiquitin chain formation through lysine 48 in ubiquitin is a canonical signal for targeting substrate proteins for proteasome-mediated degradation (9).   Therefore, these data indicate that K48-linked chain form on PXR, likely directing it to the 26S proteasome for ligand- or
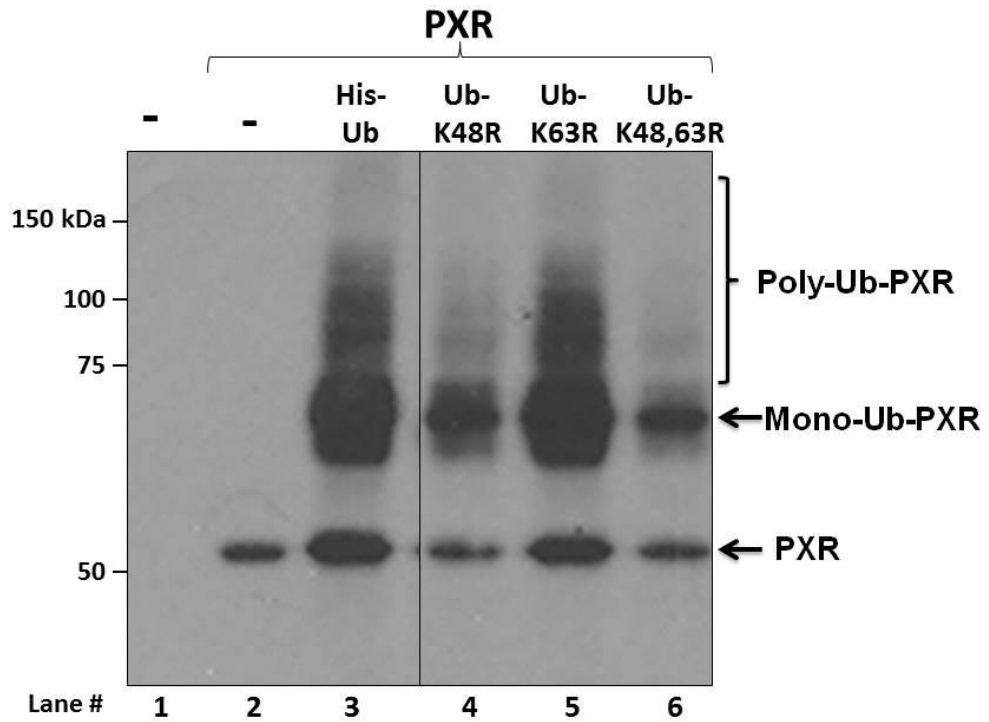
Figure 3-2

Figure 3-2. Characterization of PXR Ubiquitination. Hepa1-6 cells were transfected with plasmids encoding PXR and indicated ubiquitin mutants. 48 hours after transfection, cells were lysed using denaturing buffer. Ubiquitinated proteins were purified by using cobalt-linked agarose beads. The blot was probed for PXR immunoreactivity (sc-48340, Santa Cruz).

signal-dependent degradation. These data are consistent with those obtained for other NRs, and fit the current paradigm that ubiquitination regulates the clearance of NRs from their target gene promoters.

The Effects of UPS on PXR Transactivation. Previously our laboratory has shown that treatment with proteasome inhibitors repressed rifampicin-inducible PXR transactivation capacity in reporter gene assays using an engineered PXR reporter gene with the xenobiotic response enhancer element (XREM) from the CYP3A4 promoter (45). Moreover, overexpression of ubiquitin in CV-1 cells also repressed rifampicin-inducible PXR transactivation in XREM reporter assays. Since the data discussed above were all generated from immortalized cell lines using engineered reporter gene, primary human hepatocytes were employed to understand how the UPS regulates endogenous hPXR target genes. Primary human hepatocytes were treated with rifampicin in the presence or absence of the proteasome inhibitor MG132 for different durations of times prior to isolation of total RNA. The expression levels of CYP3A4, a well-characterized human PXR target gene, were determined by RT-qPCR. As shown in Fig. 3-3, 3 hours of drug treatment had no significant effect on CYP3A4 expression. When cells were treated for 6 hours and 18 hours, rifampicin treatment significantly increased CYP3A4 expression. Co-treatment with MG132 decreased the expression of CYP3A4 at these time points, suggesting the inhibition of 26S proteasome impaired transactivation ability of PXR. These data provide an additional link between NR-mediated gene transcription and the UPS.

The Crosstalk between Ubiquitination and SUMOylation. Different post-translational modifications are well-known to crosstalk with each other, as previously
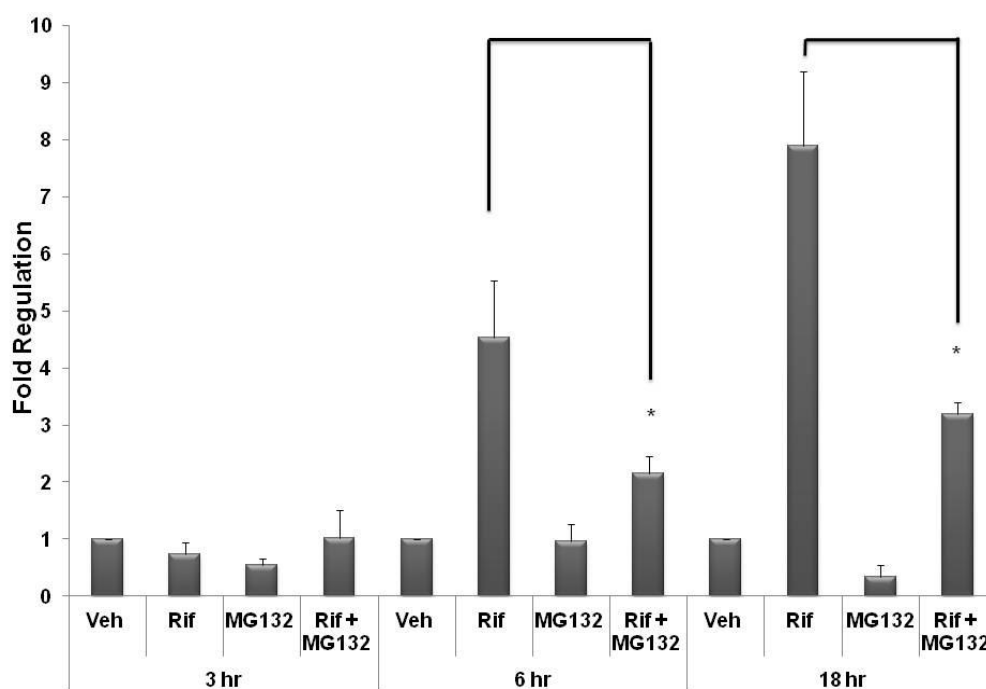
Figure 3-3

Figure 3-3.   MG132 Inhibits the Expression of CYP3A4 in Primary Cultures of Human Hepatocytes.   Primary cultures of human hepatocyte were treated with vehicle (0.1% DMSO), Rif (10μM), MG132 (10μM), or Rif together with MG132 for indicated time points. Total RNA was isolated and RT-qPCR analysis was performed to determine the expression level of CYP3A4 under different treatments.   All data are normalized to β-actin levels and data are expressed as fold regulation compared with that observed in vehicle-treated group at each time point.   Asterisks indicate a statistical difference (n=3, and $p < 0.05$).

discussed in Chapter 1.   Interaction between ubiquitination and SUMOylation at the level of PXR has been postulated, but the molecular mechanism and the biological consequence of such an interaction has not been well-described.   We next sought to determine whether promoting SUMOylation of PXR affects its ubiquitination status.   As shown in Fig. 3-4A, transfection of Hepa1-6 cells with expression vectors encoding $(His)_6$-ubiquitin and PXR produced detectable forms of ubiquitinated PXR in the absence and presence of the PXR ligand, PCN.   Transfection with PIASy, the identified E3 that promotes SUMOylation of PXR, together with either non-His-tagged SUMO1 or SUMO3 produced increased levels of unmodified PXR.   No SUMOylation of PXR was detected here because neither SUMO1 nor SUMO3 was His-tagged, and they could not be pulled down by talon metal affinity resins. When $(His)_6$-tagged ubiquitin was transfected together with PIASy and either SUMO1 or SUMO3 expression vectors, ubiquitination of PXR was dramatically increased.   These data indicate that PIASy-mediated SUMOylation of PXR stabilizes the protein, likely through prevention of ubiquitin-mediated degradation.   Examination of total protein ubiquitination using an anti-HA antibody detected increased ubiquitination levels in response to expression of PIASy together with SUMO1 or SUMO3, as shown in Fig. 3-4B.

3.5 Discussion

Ubiquitination influences the functions of target proteins by either affecting their stability or creating new surfaces for protein-protein interactions to endow them

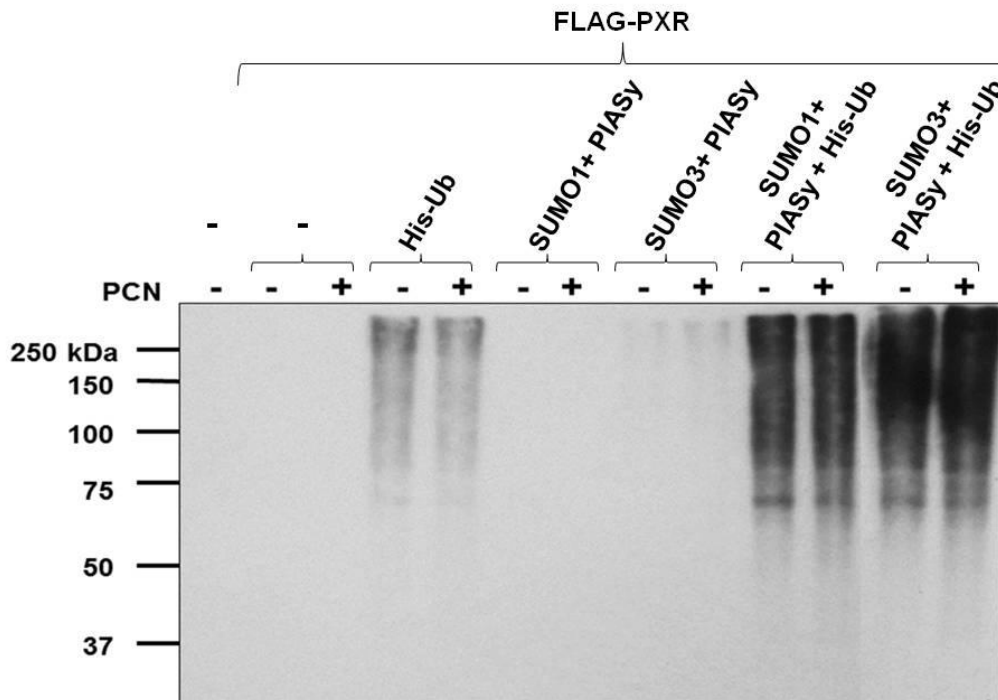**Figure 3-4**

A
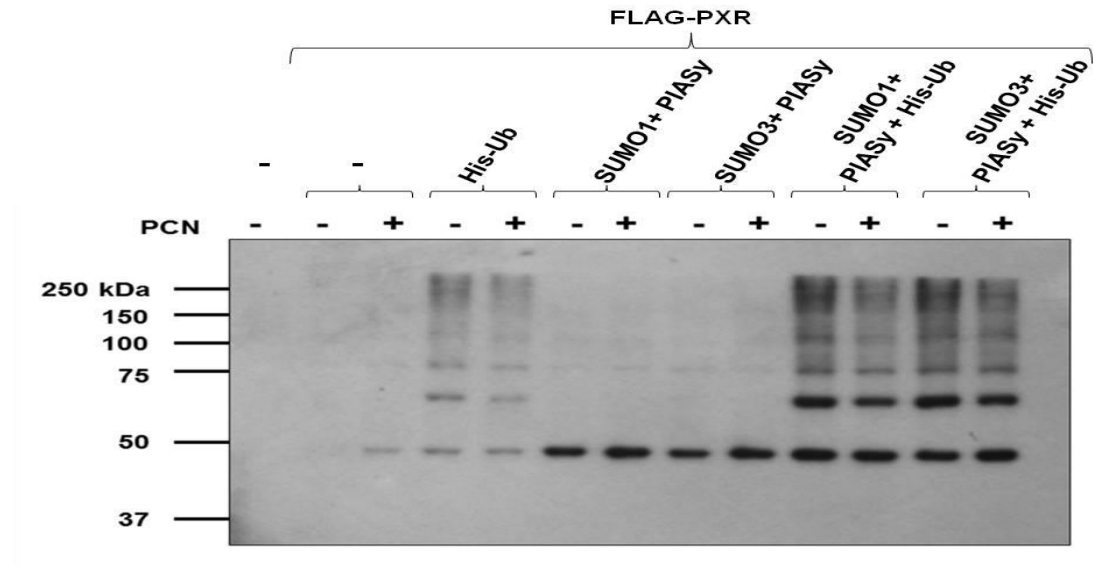
B

Figure 3-4. Crosstalk between Ubiquitination and SUMOylation in Hepa1-6 Cells.

Hepa1-6 cells were transfected with indicated plasmids. While ubiquitin is (His)$_6$-HA-tagged, both SUMO1 and SUMO3 are non-His-tagged. After transfection, cells were treated with PCN, a PXR ligand, for 24 hours, and then lysed using denaturing buffer. Ubiquitinated proteins were purified by using cobalt-linked agarose beads. The blots were probed with an anti-FLAG antibody to detect PXR immunoreactivity (PA1-984B, Thermo Scientific) (A), and an anti-HA antibody (MMS-101P, Covance) to detect ubiquitin immunoreactivity (B).

with additional signaling properties. Because of the broad range of substrates and processes in which ubiquitination is involved, aberrations in the UPS have been implicated in the pathogenesis of many diseases. The proteasome inhibitor bortezomib is approved by the FDA for the treatment of multiple myeloma and mantle cell lymphoma. Well-known for their roles in protein destruction, the contribution of UPS to transcription and the following protein synthesis was unexpected. However, accumulating studies support that the proteasome and its subunits are closely involved in transcriptional regulation, indicating that besides protein destruction, the 26S proteasome houses diverse roles in different processes. NR-mediated transcriptional regulation is complicated and subjected to multiple levels of control. Besides the processes discussed above, the UPS can also affect NR-mediated transcription through regulating chromatin structures and the destruction of co-regulatory proteins. The role of UPS in transcription is still under active investigation and many questions remain unknown.

Data presented in this chapter further confirmed that PXR is a substrate for the UPS and that K48 is the primary site of chain formation on ubiquitin that was attached to PXR protein. Inhibition of 26S proteasome impairs rifampicin-induced expression of endogenous PXR target genes. These data are consistent with those obtained for other NRs, and fit the current paradigm that ubiquitination regulates the clearance of NRs from their target gene promoters. We hypothesize that ligand-mediated activation of PXR signals PXR for destruction by the 26S proteasome. The cleared PXR-target promoter is then ready for another round of transcription, and the cycle begins anew.

Our data also indicate that the presence of SUMO increases not only the ubiquitination of PXR but also the global ubiquitination. This is in accordance with a recent publication that indicates SUMOylation can affect function of the proteasome. Psmd1, a subunit of the 19S regulatory particle, is a substrate for SUMOylation. SUMOylation of Psmd1 alters the composition and function of proteasome and impacts the degradation of proteasomal targets (46). Overexpression of SUMO1/3 in our study might have an effect on proteasomal degradation and lead to accumulation of ubiquitinated proteins, including ubiquitinated PXR. While these findings demonstrate a possible mechanism for regulation of ubiquitin-mediated protein degradation by SUMOylation, more research is needed to fully understand the crosstalk. Other underlying mechanisms may also exist. For instance, the presence of SUMO proteins might facilitate the formation of heterologous SUMO-ubiquitin chains. SUMO-target ubiquitin ligases, which specifically recognize SUMOylated proteins, might be highly activated to catalyze ubiquitination in the presence of SUMOs.

The degradation of a number of NRs by the UPS is connected to their phosphorylation status. Phosphorylation is thought to signal substrate recognition by the enzymes in the UPS. A model of phosphorylation-dependent ubiquitination has been proposed (47). PXR has been suggested as a phosphoprotein and its phosphorylation status can be modulated by the activation of PKA signaling (38, 48). The previous findings in our laboratory indicate that ubiquitination of PXR can be stimulated by treatment with cAMP, suggesting crosstalk between phosphorylation and ubiquitination. PXR has also been shown to be a substrate for acetylation (45). Whether these PTMs of PXR compete for the same lysine residues, how SUMOylation, ubiquitination, and acetylation crosstalk with each other, would different

physiological and pathological conditions alter these PTMs, whether the regulation is cell-type specific, many questions remain to be answered and the data are just beginning to emerge. Future studies should include an examination of the potential interaction between different signaling pathways and PTMs at the level of the PXR protein.

The data presented here demonstrate that the UPS affects PXR function in regulating target gene expression. The extent to which ubiquitination and/or degradation of PXR affects glucose homeostasis, lipid homeostasis, vitamin D metabolism, and inflammation in mammals requires further investigation. The activity of PXR is affected by multiple signaling pathways and our previous studies indicate that the ubiquitination status of PXR is under the control of PKA and MEKK1 pathways. Further studies are also required to see how different physiological and pathological conditions affect the ubiquitination status of PXR. Considering the versatile roles of PXR in numerous physiological pathways, pharmacological manipulation of the complex network that contribute to the regulation of PXR activity may lead to the identification of therapeutic opportunities for treating of numerous diseases.

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Chapter 4: SUMOylation/DeSUMOylation of PXR and Their Roles in the Regulation of Inflammatory Response

4.1 Introduction

Highly expressed in the liver and intestine, PXR was discovered in 1998 based on the structural homology to other nuclear receptors (1). As a ligand-dependent transcription factor of the nuclear receptor superfamily, PXR plays an important role in regulating xenobiotic metabolism and clearance. PXR forms a heterodimer with RXRα and binds to PXR response elements found mostly within the promoter region of its target genes. Unlike other NRs, the ligand binding domain of PXR is relatively flexible and large, which can accommodate the binding of numerous structurally-diverse molecules. Thus, PXR can be activated by many different xenobiotics as well as endobiotics, including endocrine disrupting compounds, drugs and bile acids. Once activated, PXR up-regulates the expression of genes encoding phase I (oxidation), phase II (conjugation) metabolizing enzymes and phase III transporters to increase metabolism and clearance of xenobiotics from the body, protecting the body from potential toxic insults (2). However, activation of PXR is not without a risk. As a key regulator of drug metabolism and clearance, unexpected or abnormal activation of PXR may lead to adverse drug-drug and disease-drug interactions, which are major clinical problems increasing not only medical costs but also morbidity and mortality (3). In addition to its roles in xenobiotic metabolism and clearance, PXR also has a regulatory effect on inflammation, glucose and lipid metabolism, bile acid and bilirubin detoxification, steroid hormones and vitamin metabolism. Therefore, PXR activation has important implications in

many patho-physiological conditions. Recent studies indicate that activation of PXR is beneficial in the treatment of diseases like cholestasis, inflammatory liver disease and inflammatory bowel diseases, suggesting the potential of PXR as a therapeutic target (4-6).

Inflammation is associated with many diseases in liver and intestine. It is of great importance to regulate inflammatory response. As early as 40 years ago, before PXR was discovered, it was noticed that treatment with rifampicin, the prototypical human PXR ligand, could suppress humoral and cellular immunological response (7). Nowadays, it is well-accepted that activation of PXR has a negative regulatory role on inflammatory response. However, the underlying mechanism is not fully understood. Multiple studies demonstrate that this phenomenon is partially due to the crosstalk between PXR and NF-κB signaling. However, the precise molecular details involved have not been fully established and additional pathways which are not mutually exclusive might exist to regulate this process.

Post-translational modifications (PTMs) are involved in the dynamic regulation of protein functions. Among different PTMs, SUMOylation has gained more and more attention as it is closely associated with many cellular activities, including cell cycle progression, genome integrity, and signal transduction. Moreover, SUMOylation also regulates the activities of NRs. In a historical perspective, many components of the SUMO machinery were first identified as nuclear receptor-associated proteins before their recognition as SUMO machinery components (8, 9). PXR, together with many other NRs, are substrates for SUMOylation and their activities are regulated by SUMOylation status as discussed in chapter 1.

SUMOylation involves the covalent binding of SUMO proteins to select lysine(s) within substrates. There are four SUMOs in mammals, SUMO1-4. However, SUMO4 is not processed for SUMOylation, and its function still remains unclear (10). Among other SUMOs, SUMO2 and SUMO3 are similar to each other, whereas SUMO1 is distinct from them (11). SUMO2 and 3 are well-characterized to form SUMO chains through lysine 11 within the SUMO2/3 proteins (12, 13). Though it is generally considered that SUMO1 lacks the endogenous SUMOylation site to form chains, SUMO1 chain formation has also been observed (14). Just like ubiquitination, SUMOylation also requires a cascade of enzyme activities. SUMO proteins are first translated as precursors and a few amino acids at the C terminus are cleaved off by sentrin-specific proteases (SENPs) to expose the di-glycine motif and form mature SUMOs. The mature SUMOs can be activated by SUMO activating enzyme, E1, in an ATP-dependent manner and transferred to SUMO conjugating enzyme, E2 (15, 16). With the help of E3, SUMOs will be transferred to their substrates, forming the isopeptide bond. SUMOylation is a dynamic and reversible process. SENPs can remove SUMOs from substrates and this process is known as de-SUMOylation. During the SUMOylation cycle, SENPs carry out two main functions: facilitating SUMO maturation as an endopeptidase and deconjugating SUMO from substrates as an isopeptidase. Since only one set of E1 (SAE1/SAE2) and one E2 (UBC9) have been identified, it is believed that E3 and SENPs specify SUMOylation substrates. PIAS (protein inhibitor of activated STAT) family is one of the best characterized SUMO E3 ligase families. PIAS proteins have RING finger domains, which assist in the attachment of SUMO to target substrates. Unlike Ubc9, which forms a thioester bond with SUMO, PIAS proteins bind to SUMO non-covalently to

facilitate SUMOylation (17). For de-SUMOylation, there are 6 SENPs identified in human which are involved in the deconjugating pathway, which are SENP1, 2, 3, 5, 6 and 7. Different SENPs have varied localizations in cells and have different specificity towards substrates (18).

SUMOylation regulates a plethora of proteins in many cellular processes. SUMOylation of transcription factors, in most cases, is correlated with transcriptional repression, even though exceptions exist. For instance, SUMOylation of T-cell factor-4 (TCF-4) is suggested to increase its transcriptional activities (19). SUMOylation of transcription factors can lead to new interaction interfaces on transcription factors that promote the recruitment of corepressor complexes. Using different transcription factors, it is shown that histone modifying enzymes like histone deacetylases (HDACs), histone demethylases and histone methyltransferases can be recruited to transcription factors in a SUMOylation dependent manner. Through the regulation of histone modifications, recruited enzymes alter the chromatin structure and actively repress gene expression (20). In addition to recruiting corepressor complexes, other mechanisms have also been proposed. For example, SUMOylation can compete with other post-translational modifications, regulate the subcellular localization of transcription factors, prevent some NRs from efficiently dimerizing with RXRs, and inhibit the DNA-binding of transcription factors (21).

Besides transcription factors, many co-regulatory proteins in transcription process are also SUMOylated. SUMOylation can increase the deacetylase activity and transcriptional repressor activity of HDAC1 and HDAC4 (22, 23). SUMOylation of SRC-3, a coactivator

for transcription factors, leads to transcriptional attenuation at responsive genes (24). These

lead to another layer of complexity in SUMOylation-mediated transcriptional regulation.

While SUMOylation can directly modify transcription factors, it can also regulate

transcriptional activity through transrepression. Many NRs have been suggested to be

SUMOylated to repress inflammatory responses, including LXRs and PPARγ. SUMOylated

LXRs and PPARγ are recruited to promoters of TLR (Toll-like Receptor) target genes, where

they prevent the removal of NCoR corepressor complexes and inhibit the expression of

inflammatory response genes (25). Previous work from our laboratory suggests that

SUMOylation is also involved in human PXR mediated repression of inflammatory response

genes (26).

In this study, we extend our previous observation and further characterize

SUMOylation/deSUMOylation of PXR and their potential roles in regulating PXR activity.

We also demonstrate that activation of PXR inhibits the expression of specific LPS-inducible

inflammatory genes in both primary hepatocytes and immortalized cell lines and further prove

that SUMOylation is responsible for PXR-mediated repression of inflammatory response.

4.2 Materials and Methods

Plasmids and Chemicals. The full length mouse PXR expression vector,

CMV-mPXR, was previously described (1). pcDNA4-HisMax-SUMO1 was subcloned

from pcDNA3-SUMO1 utilizing BamHI site. pcDNA4-HisMax-SUMO3 was subcloned

from pcDNA3-SUMO3 using BamHI and EcoRI sites. Expression vectors for PIASs, SENPs and corresponding mutants were obtained from Addgene. All chemicals were purchased from Sigma and all cell culture supplements were purchased from Gibco unless otherwise indicated.

Primary Hepatocyte Culture. PXR-KO mice and hPXR$_{tg}$ mice were generated as previously described (27, 28). Primary mouse hepatocytes were isolated from male congenic (C57BL6) wild type, PXR knockout (KO) and humanized PXR-transgenic (hPXR$_{tg}$) mice using a standard collagenase perfusion method. In brief, the liver was cleared with wash buffer (1×HBSS, 0.5mM EGTA, 5.5mM glucose) at a flow rate of 6 ml/min for 8 min, and then digested for 8 min with digestion buffer [1×HBSS, 1.5 mM CaCl$_2$, 5.5 mM glucose, ~160 U/ml collagenase II (Worthington)] at a flow rate of 6 ml/min. After digestion, the liver was excised and punctured open using forceps in digestion buffer to release hepatocytes. The cell suspension was filtered through a 100-μm nylon cell strainer (BD Falcon). Cells were pelleted at 600 rpm for 5 min and washed once in plating medium (Dulbecco's Modified Eagle's Medium supplemented with 5% fetal bovine serum, 100 nM dexamethasone, 1 μM insulin, 2 mM L-Glutamine, 100U/ml penicillin G, and 100μg/ml streptomycin). Dead cells were separated by centrifugation at 1000 rpm for 5 min in 30% Percoll (Sigma). After one more wash in plating medium, cell viability was determined using trypan blue staining. Hepatocytes were plated in 6-well collagen-coated cell culture plates at a density of 7.0×10^5 living cells/well in plating medium. After overnight attachment at 37°C in a humidified incubator with 95% air and 5% CO$_2$, plating medium was replaced with William's Medium E supplemented with 0.1 mg/ml Matrigel (BD Bioscience), 100 nM dexamethasone, 0.1 mg/ml

Matrigel (BD Bioscience), 2 mM L-Glutamine, 1×ITS, 100U/ml penicillin G, and 100μg/ml streptomycin.

The primary human hepatocytes were derived from samples collected and provided by the University of Kansas Medical Center, Department of Pharmacology, Toxicology and Therapeutics Hepatocyte Core Lab and the KU Liver Center which is sponsored by the Department of Pharmacology, Toxicology and Therapeutics Biospecimen Core Lab and the Liver Center at KUMC. Freshly isolated human hepatocytes were plated at a cell density of 5×10^5 cells/well in 12-well plates previously coated with 0.2 mg/ml type I collagen. Isolated hepatocytes were maintained in Dulbecco's Modified Eagle's Medium supplemented with 100 nM dexamethasone, 100 nM insulin, 100U/ml penicillin G, 100μg/ml streptomycin, and 5% bovine calf serum and kept at 37℃ in a humidified incubator with 95% air and 5% CO_2. Hepatocytes were allowed to attach to the plate for 4 hours and then the medium were changed to William's Medium E supplemented with 100 nM dexamethasone, 0.1 mg/ml Matrigel (BD Bioscience), 2 mM L-Glutamine, 1×ITS, 100U/ml penicillin G, and 100μg/ml streptomycin.

Total RNA Isolation, Reverse Transcription, and Real-Time Quantitative-Polymerase Chain Reaction Analysis. Total RNA was isolated from cells using the commercially available RNeasy Mini Kit (Qiagen) according to the manufacturer's instructions. 2μg of RNA was reverse transcribed using random primers (Promega). Reverse transcription cycling conditions were 25℃ for 10 min, 42℃ for 60 min, and 95℃ for 5 min. Equal amounts of cDNA were used in real-time quantitative polymerase chain

reactions (RT-qPCRs). Reactions included 1 X Power SYBR Green (Applied Biosciences) and 300nM primers specific for each gene. The sequences of each primer are as follows:

Genes	Primers
β-actin	Mouse: 3' primer: 5'-TAACAGTCCGCCTAGAAGCA-3' 5' primer: 5'-CAAGATCATTGCTCCTCCTG-3' Human: 3' primer: 5' CAAGATCATTGCTCCTCCTG 3' 5' primer: 5' TCATAGTCCGCCTAGAAGCA 3'
IL-6	Mouse: 3' primer: 5'-CTGCAAGAGACTTCCATCCAG-3' 5' primer: 5'-AGTGGTATAGACAGGTCTGTTGG-3' Human: 3' primer: 5'-ACTCACCTCTTCAGAACGAATTG-3' 5' primer: 5'-CCATCTTTGGAAGGTTCAGGTTG-3'
IL-1β	Mouse:

	3' primer: 5'-GAAATGCCACCTTTTGACAGTG-3'
	5' primer: 5'-CTGGATGCTCTCATCAGGACA-3'
	Human:
	3' primer: 5'-ATGATGGCTTATTACAGTGGCAA-3'
	5' primer: 5'-GTCGGAGATTCGTAGCTGGA-3'
IL-1Ra	Mouse:
	3' primer:5'-TAGACATGGTGCCTATTGACCT-3'
	5' primer:5'-TCGTGACTATAAGGGGCTCTTC-3'
	Human:
	3' primer: 5'-CATTGAGCCTCATGCTCTGTT-3'
	5' primer: 5'-CGCTGTCTGAGCGGATGAA-3'
Cox-2	3' primer: 5'-TTCCAATCCATGTCAAAACCGT-3'
	5' primer: 5'-AGTCCGGGTACAGTCACACTT-3'
Cyp3a11	3' primer: 5'-CCACGTTCACTCCAAATGAT-3'
	5' primer: 5'-CAAGGAGATGTTCCCTGTCA-3'
CYP3A4	3' primer: 5'-CAGGAGGAAATTGATGCAGTTTT-3'

	5'primer: 5'-GTCAAGATACTCCATCTGTAGCACAGT-3'

RT-qPCR was performed using the StepOnePlus Real-Time PCR System (Applied Biosystems).   Cycling conditions were 95 ℃ for 10 min followed by 45 cycles of 95 ℃ for 10s and 60°C for 1 min.   All data were normalized to β-actin and fold induction was calculated using the $\Delta\Delta^{ct}$ method.

NF-κB Gene Array.   Total RNA was isolated from wild type mouse hepatocytes using the RNeasy Mini Kit (Qiagen) and reverse transcribed.   The commercially available Mouse NF-κB Gene Array (SA Biosciences, 330231 PAMM-225A) was purchased and an equal amount of cDNA was applied to each well before RT-qPCR was performed as described above.

Analysis of IL-1Ra.   A 200 μl aliquot of cell media was removed and combined with 200 μl 2×SDS-PAGE loading buffer supplemented with 50mM DTT.   Following removal of culture media, cells were harvested by scraping into 1×PBS, pelleted by centrifugation, and then lysed in SDS-PAGE loading buffer supplemented with DTT.   Equal amounts were resolved using 12.5% SDS-PAGE.   Western blot analysis was performed using a monoclonal antibody that recognizes human and mouse IL-1Ra (NBP1-96673, Novus Biologicals).   Western blot images were quantified by densitometric scanning of X-ray films with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software.

Cell-based SUMOylation Assay.   Hepa1-6 cells were transfected using Lipofectamine 2000 according to the manufacturer's instructions.   48 hours after transfection, cells were harvested in lysis buffer (6M guanidinium-HCl, 10mM Tris, 100mM sodium phosphate buffer, pH 8.0), sonicated, the cell lysates cleared by centrifugation and then mixed with 30μl of Talon metal affinity resins (Clontech) equilibrated in lysis buffer.   The mixture was

incubated with rotation for 2 hours at room temperature and washed twice in lysis buffer, three times in Urea 6.5 buffer [8M urea (Fisher Scientific), 10mM Tris, and 100mM sodium phosphate buffer, pH 6.5, supplemented with 20mM imidazole (Fisher Scientific)] and once in 1×PBS.   After final wash, the affinity resins were resuspended in 30μl of 2×SDS-PAGE gel loading buffer supplemented with 50mM DTT, boiled for 5 min, and the proteins resolved using 10% SDS-PAGE.   The gel was transferred to polyvinylidene difluoride membrane and probed with anti-FLAG antibody (PA1-984B, Thermo Scientific).

Statistical Analyses.   Where appropriate, statistical differences among experimental groups were determined using a one-way analysis of variance followed by the Duncan's multiple range post hoc test.   Letters different from each other indicate a significant difference between treatment groups.   Statistical differences between experimental groups were determined using the student's t-test.

4.3 Results

4.3.1 The Role of PXR in Modulating the Inflammatory Response in Primary Cultures of Hepatocytes

LPS-Inducible Concentration- and Time-responses Analysis of Key Inflammatory Mediators in Mouse and Human Primary Cultures of Hepatocytes.   To determine the extent to which activation of PXR alters LPS (lipopolysaccharide)-inducible gene expression, concentration- and time-responses of LPS-inducible IL-1β (interleukin-1β) gene expression were examined in mouse and human hepatocytes.   Treatment of hepatocytes with increasing

amounts of LPS (0.01, 0.1, 1, 10, and 100 μg/ml) for 12 hours produced robust induction of IL-1β expression at all concentrations examined (Fig. 4-1A). Based on these results, 10 μg/ml was chosen to initiate the inflammatory response in subsequent studies. While all of the time points examined (1, 6, 12, 24, and 48 hours) exhibited significant increases in IL-1β expression level, 6 and 12 hour time points showed the largest increases (Fig. 4-1B). Examination of kinetics of LPS-inducible mouse and human IL-6 (interleukin-6) produced very similar results (data not shown). Based on these data, 12 hours treatment was chosen to induce the expression of key inflammatory mediators in both mouse and human hepatocytes. A time-response analysis of CYP3A gene expression using PCN (10μM) as a prototypical mouse PXR activator and rifampicin (10μM) as a prototypical human PXR activator indicated that 24 hours treatment produced maximal CYP3A gene expression (Fig. 4-2). Taken together, pre-treatment with PXR activators for 24 hours and subsequent co-treatment with LPS for 12 hours was selected for subsequent experiments to assess the effect of PXR activation on LPS-inducible gene expression.

Pre-Activation of PXR Suppresses LPS-inducible NF-κB Target Genes. An important transcriptional mediator of LPS signaling is the transcription factor NF-κB. To determine the effect of PXR activation on the inflammatory response in primary mouse hepatocytes, a commercially available NF-κB PCR array was employed. This array allows us to simultaneously examine the expression of 84 key genes which are responsive to NF-κB signal transduction. Based on previous dose and time experiments, hepatocytes were pre-treated with PCN or vehicle for 24 hours. Cell cultures were then divided into four
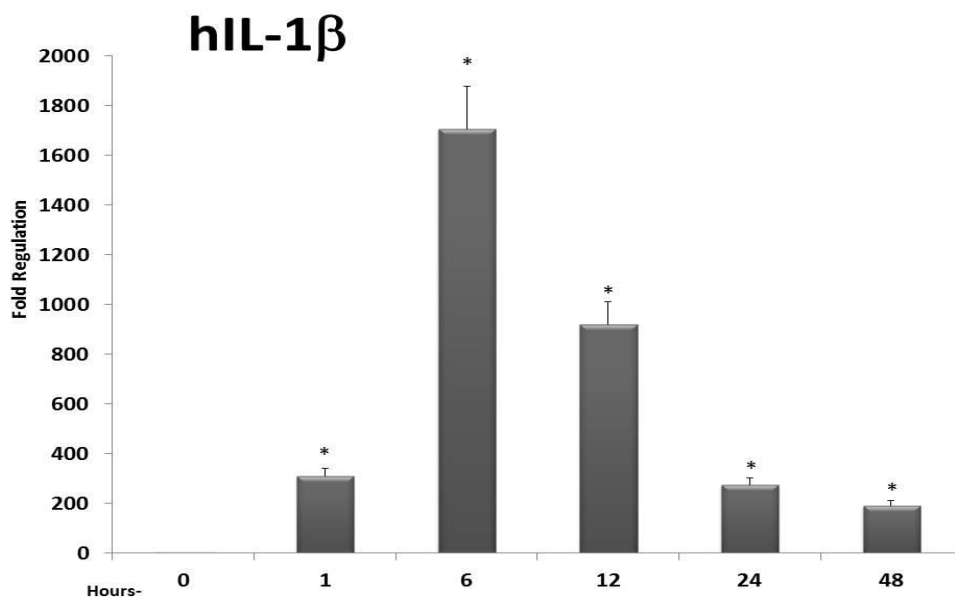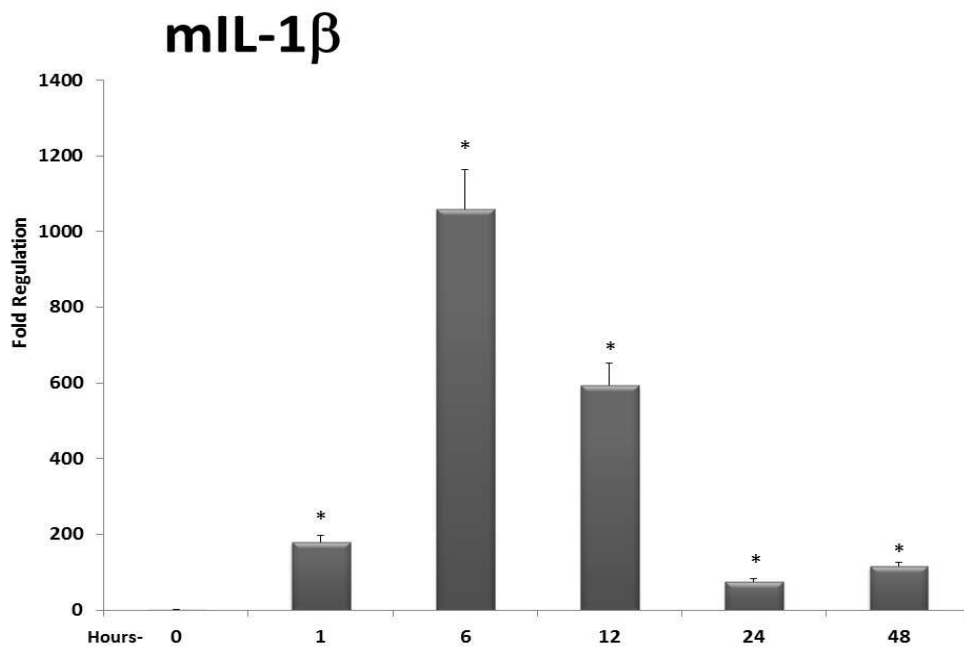
Figure 4-1

A

B

mIL-1β

hIL-1β
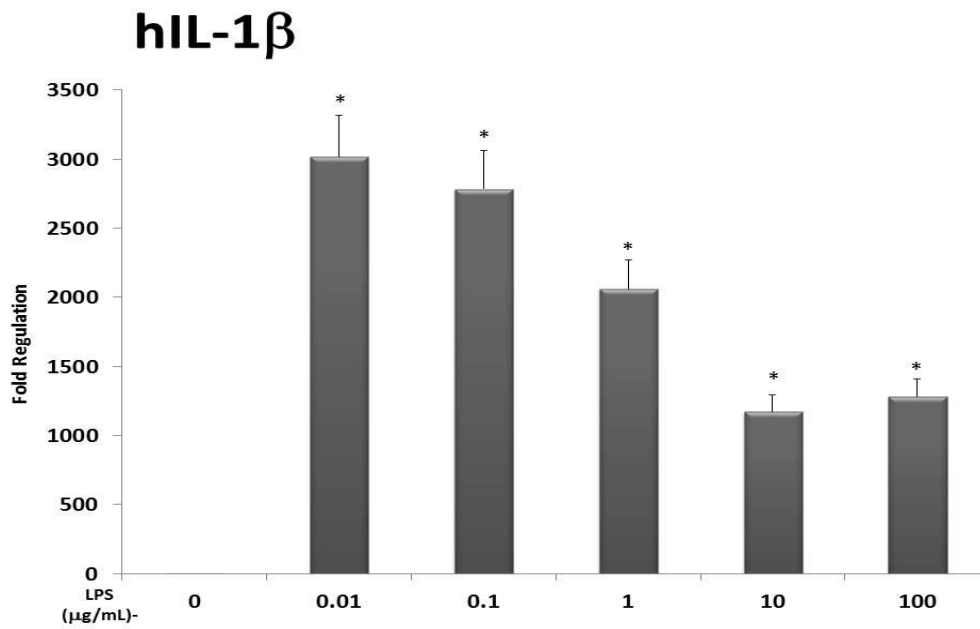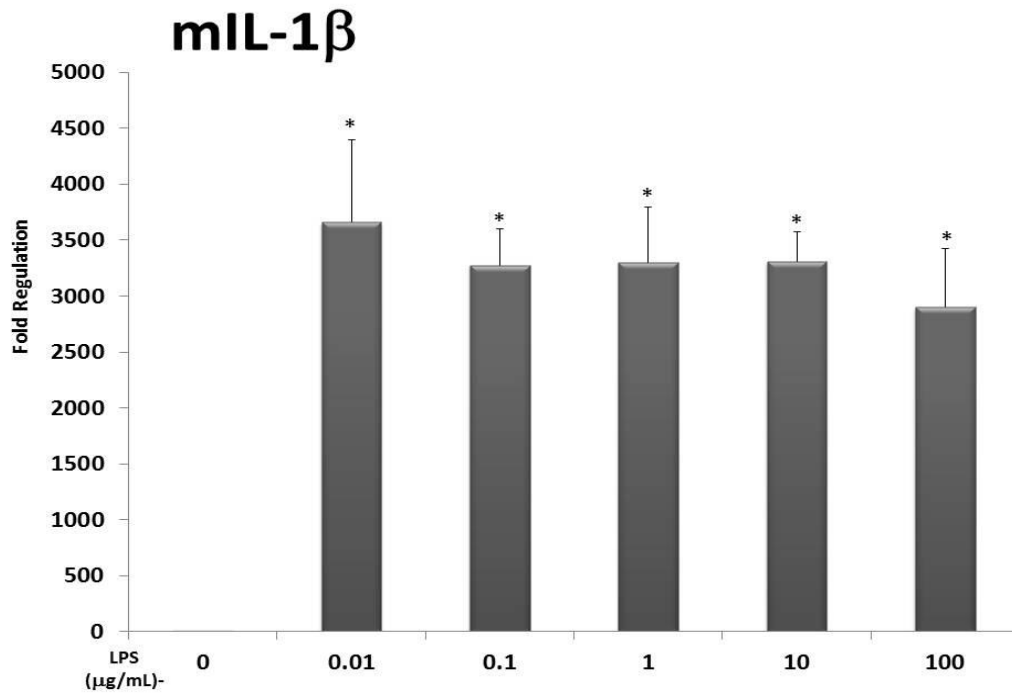
Figure 4-1. Concentration- and Time-dependent Analysis of the Expression of Inflammatory Response Genes in Hepatocytes. (A) Primary cultures of wild type mouse hepatocytes or human hepatocytes were treated with vehicle or indicated concentrations of LPS for 12 hours. Total RNA was isolated and RT-qPCR analyses were performed to determine the expression of IL-1β under different concentrations of LPS treatment. (B) Primary cultures of wild type mouse hepatocytes or human hepatocytes were treated with vehicle or LPS (10 μg/ml) for indicated time points. Total RNA was isolated and RT-qPCR analyses were performed to detect the expression levels of IL-1β mRNA through time. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

Figure 4-2
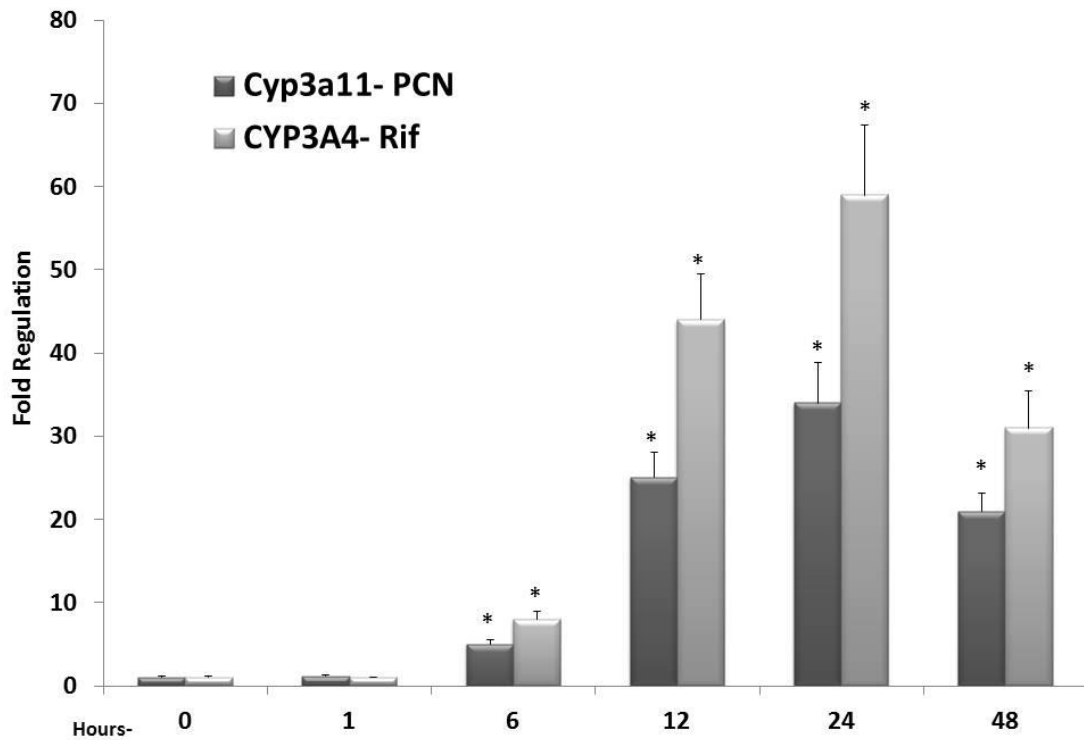
Figure 4-2. **Time-dependent Induction of CYP3A Gene Expression in Primary Hepatocytes Derived from Mice and a Human Donor.** Hepatocytes were treated with vehicle (0.1% DMSO), Rif (10 μM) or PCN (10 μM) for the indicated time points. Total RNA was isolated and the relative expression level of Cyp3a11 (mouse) and CYP3A4 (human) were determined. All data are normalized to β-actin levels and are presented as fold regulation. Asterisks indicate a statistical difference from vehicle treated samples (n=3, and $p<0.05$).

experimental groups and were treated for an additional 12 hours with either vehicle, PCN alone, 10μg/ml LPS alone, or PCN and LPS together. After the treatment, total RNA was isolated and reverse transcribed. cDNA was subjected to the PCR array. As shown in Table 4-1, treatment of primary mouse hepatocytes with LPS for 12 hours induced the expression of sixteen well-known NF-κB target genes. Treatment with PCN alone repressed the basal expression of numerous NF-κB target genes (Table 4-2). When compared with LPS treatment alone, treatment with PCN for 24 hours and subsequent co-treatment with LPS produced significantly lower expression levels of several notable LPS-inducible NF-κB target genes, including IL-1β, IL-6, Ptgs2 (also known as Cox-2, cyclooxygenase 2), and IL-1Ra (As shown in Table 4-3). These data suggest that PXR represses the inflammatory response in a gene-specific manner, since not all NF-κB target genes were repressed by PXR activation.

For the following experiments, four NF-κB target genes, Cox-2, IL-1β, IL-6, and IL-1Ra were chosen for in-depth study. To further determine the role of PXR in the repression of the inflammatory response, hepatocytes derived from wild type or PXR-KO mice were used with independently designed RT-qPCR primer sets. The integrity of PXR-KO hepatocytes was confirmed by analysis of the expression levels of Cyp3a11, an mPXR target gene. As shown in Fig.4-3A, while PCN treatment significantly induced the expression of Cyp3a11 in WT hepatocytes, there was no induction in PXR-KO hepatocytes. In accord with our previous publication, co-treatment with LPS diminished the induction of Cyp3a11 by PCN in WT hepatocytes (29). In WT hepatocytes, treatment with LPS alone significantly increased

Table 4-1

Gene Induced by LPS	Fold Induction	S.D
Cxcl3	393.9	80.8
IL-1β	314.4	77.4
IL-6	128.9	43.2
Csf3	63.4	20.4
IL12β	52.5	12.4
Ccl5	50.5	11.5
Ptgs2	50.5	10.6
Ltb	50.0	10.6
IL-1Ra	49.7	9.2
IL-1α	31.0	7.8
Cxcl1	10.2	2.3
TNFα	10.0	4.6
Sele	6.3	2.9
Cd74	6.3	2.1
Vcam1	5.0	1.1
Bcl2a1a	3.9	1.3

Table 4-1. Treatment of Primary Cultures of Mouse Hepatocytes with LPS Increases Expression of NF-κB Target Genes. Primary hepatocytes isolated from wild type mice were treated for 12 hours with either vehicle (0.09% saline) or LPS (10μg/ml) (n=4). Total RNA was isolated and RT-qPCR using a focused panel of 84 well-known NF-κB target genes was performed following manufacturer's instructions (SA Biosciences). Data are expressed as fold induction $\pm$ standard deviation (S.D.) where $p \leq 0.05$.

Table 4-2

Genes Suppressed by PCN	Fold Suppression	S.D.
Selp	5.1	2.4
C3	4.2	2.0
Csf2	4.1	0.3
Tnfsf10	4.1	2.0
Agt	4.1	1.9
Myd88	3.3	1.4
Aldh3a2	3.3	1.6
Csf2rb	3.2	1.5
F8	3.2	1.5
Ifnb1	2.6	1.0
Cfb	2.6	1.0
Il1rn	2.5	0.8
Ifnɣ	2.2	0.8
Trp53	2.1	0.8
Akt1	2.1	0.8
Ccl22	2.1	0.8
Nqo1	2.1	0.8
Cxcl3	2.1	0.6

Mitf	2.0	0.8
Fas	2.0	0.8
Stat3	2.0	0.8
Tnfrsf1b	2.0	0.8
Rel	2.0	0.8
Stat5b	2.0	0.8
Xiap	2.0	0.8
Irf1	2.0	0.8
Fasl	2.0	0.7

Table 4-2.   Treatment of Primary Cultures of Mouse Hepatocytes with PCN Suppresses Basal Expression of NF-κB Target Genes. Cultures of mouse hepatocytes isolated from wild type mice were treated with either vehicle (0.1% DMSO) or 10 μM PCN for 24 hours (n=3). Total RNA was isolated and reverse transcribed. RT-qPCR using a focused panel of 84 well-known NF-κB target genes was performed following manufacturer's instructions (SA Biosciences). Data are reported as fold suppression $\pm$ the standard deviation (S.D.) in the PCN treated group when compared to vehicle treated cells ($p \leq 0.05$)

Table 4-3

Genes Suppressed in [LPS + PCN] -vs- LPS Alone	Fold Suppression	S.D.
Ptgs2	2.5	0.6
Mmp9	2.5	0.8
Cd83	2.5	0.8
Cd74	2.5	0.6
Il6	2.0	0.4
Il1rn	2.0	0.5
Il1β	1.6	0.2
Tnfα	1.6	0.1

Table 4-3.   Treatment of Primary Cultures of Mouse Hepatocytes with PCN Suppresses LPS-induced Expression of NF-κB Target Genes.   Cultures of mouse hepatocytes isolated from wild type mice were pre-treated with vehicle or 10 μM PCN for 24 hours then co-treated with LPS for another 12 hours (n=3).   Total RNA was isolated and reverse transcribed.   RT-qPCR using a focused panel of 84 well-known NF-κB target genes was performed following manufacturer's instructions (SA Biosciences).   Data are reported as the average fold suppression $\pm$ the standard deviation (S.D.) in the co-treated (PCN+ LPS) group when compared with LPS alone ($p \leq 0.05$).

Figure 4-3

A
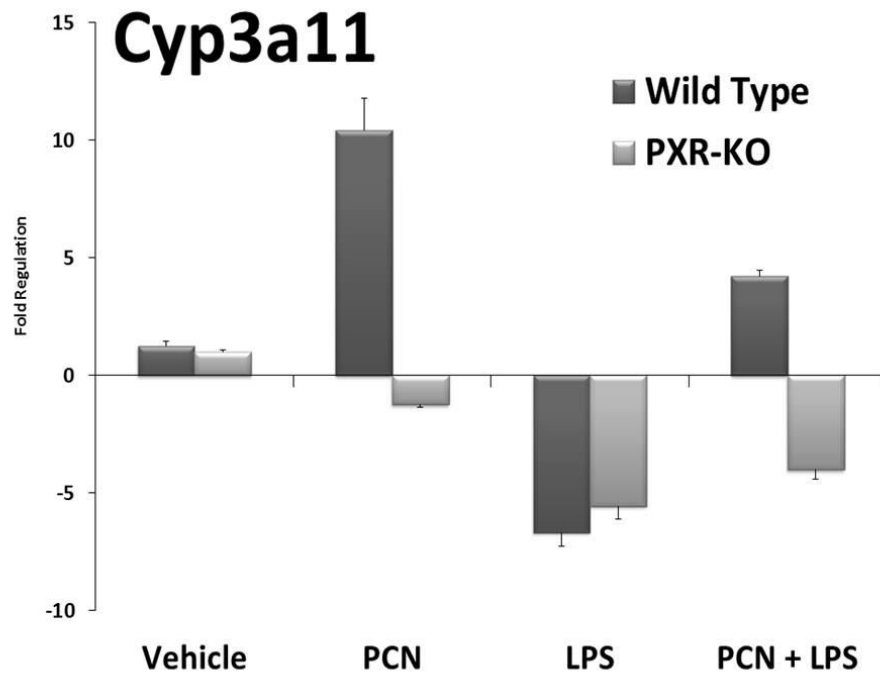
B
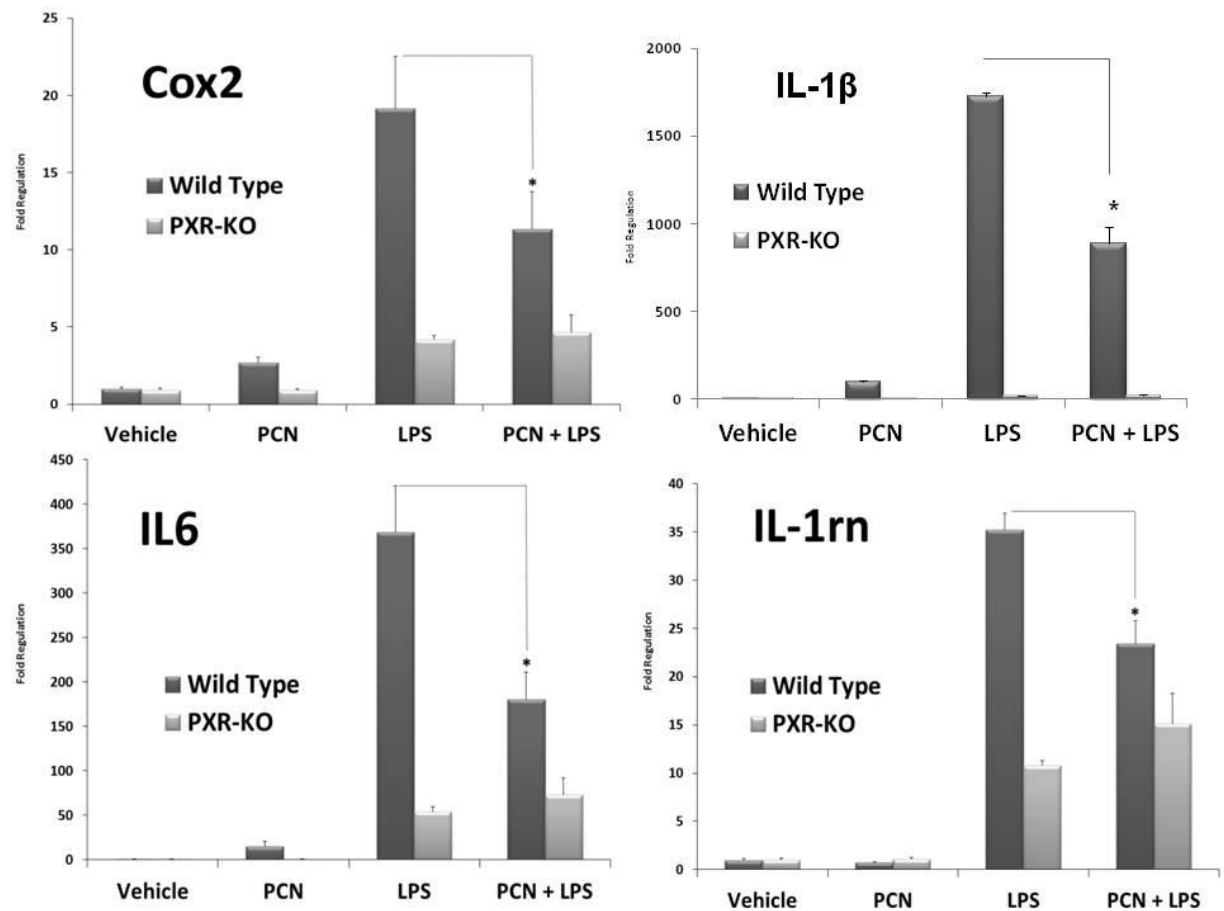
Figure 4-3.   PXR Activation Represses the Expression of LPS-inducible Inflammatory

Response Genes.   Primary hepatocytes were isolated from wild type (C57BL/6) or

PXR-KO mice.   After overnight attachment, cells were treated with vehicle (0.1% DMSO)

or 10 μM PCN for 24 hours followed by an additional 12 hours treatment with or without LPS

(10 μg/ml).   Total RNA was isolated, and the relative expression levels of (A) Cyp3a11 and

(B) Cox-2, IL-1β, IL-6 and IL-1rn were determined by RT-qPCR.   The expression level in

vehicle treated hepatocytes was set as 1 in each genotype.   All data are normalized to β-actin

levels and are presented as fold regulation (* indicates $p < 0.05$).

the expression of selected inflammatory response genes, whereas this induction was suppressed by the pre-activation of PXR with PCN, as shown in Fig. 4-3B, However, in PXR-KO hepatocytes, no suppression was observed in the co-treatment group compared to LPS treatment alone, suggesting the repression is PXR-dependent. It was noticeable that in PXR-KO hepatocytes the fold induction by LPS is much lower compared to WT hepatocytes. This can be partially explained by the fact that in PXR-KO hepatocytes, expression levels of inflammatory response genes are higher in the basal level, indicating a repressive role for PXR in regulating basal expression levels of key inflammatory mediators. When comparing all the groups to vehicle-treated wild type hepatocytes, LPS-inducible IL-1β expression level was even higher in PXR-KO hepatocytes than wild type hepatocytes (data not shown). However, this only occurred in IL-1β expression level. For other tested inflammatory genes, the LPS-inducible expression levels were still lower compared to wild type hepatocytes. These data reveal that the absence of PXR produces a condition in which the expression of IL-1β is heightened, and suggests that PXR contributes to the effective suppression of IL-1β inflammatory response through time. Moreover, in WT hepatocytes, PCN alone could induce the basal expression of Cox-2, IL-1β, and IL-6, but this induction was abolished in PXR-KO hepatocytes. While mechanisms underlying these phenomena are unknown, these data suggest that PXR plays a role in the regulation of inflammatory responses.

The Effects of PXR Activation on IL1-Ra Expression. Of all the NF-κB target genes we examined, IL-1Ra (protein product of IL-1rn gene) is of particular interest. Unlike other examined NF-κB target genes which are pro-inflammatory mediators, IL-1Ra is anti-inflammatory. IL-1Ra is expressed at high levels in hepatocytes. Its expression can be

induced by inflammatory mediators to encode a secreted antagonist of IL-1 signaling (30, 31). lL-1Ra regulates IL-1α and IL-1β activity by competing with them for binding of the IL-1 receptor. IL-1Ra can bind to IL-1 receptor with similar affinity as IL-1α and β, but it does not initiate the downstream inflammatory signaling. Through the competition with IL-1α and IL-1β, IL-1Ra antagonizes IL-1 inflammatory signaling.

To further study the effect of PXR activation on IL-1Ra expression, the concentration- and time-dependent LPS-inducible expression of IL-1Ra were closely examined in both wild type mouse hepatocytes and human hepatocytes. Treatment with LPS for 12 hours produced a significant increase in the expression level of IL-1Ra in a concentration-dependent manner (Fig. 4-4A). For the time-dependent LPS-inducible expression of IL-1Ra, the expression levels were significantly induced at 6, 12, 24, and 48 hours (Fig. 4-4B). When compared with the time- and concentration-response analysis of IL-1β and IL-6, the induction of IL-1Ra was delayed by several hours, but remained relatively high throughout the 48 hours. These data indicate that the kinetics of LPS-inducible IL-1Ra gene expression is distinct from that observed for pro-inflammatory mediators like IL-1β and IL-6, with expression levels of IL-1Ra increasing at later time points and exhibiting a longer period of sustained expression.

There are two major isoforms of IL-1Ra protein, one is a heavily glycosylated and secreted isoform (sIL-1Ra) and the other is an intracellular isoform (icIL-1Ra). The expression and secretion of sIL-1Ra is highly inducible, whereas the expression of icIL-1Ra is not. To fully examine PXR's role in IL-1Ra expression, we examined the PXR- and LPS-inducible level of sIL-1Ra and icIL-1Ra proteins in both media and whole cell lysate,

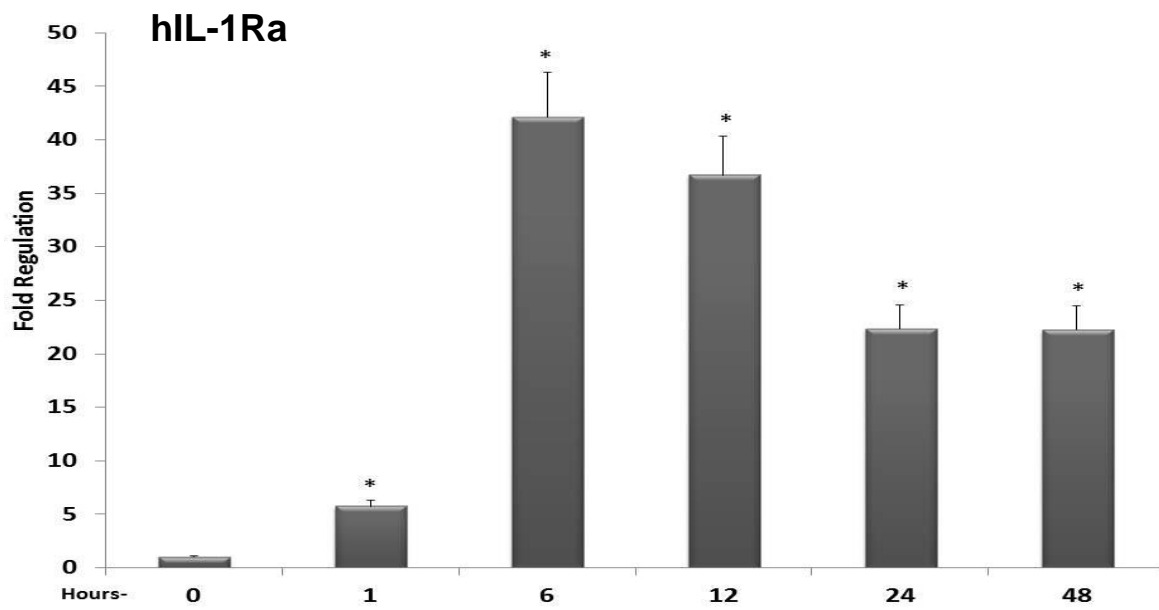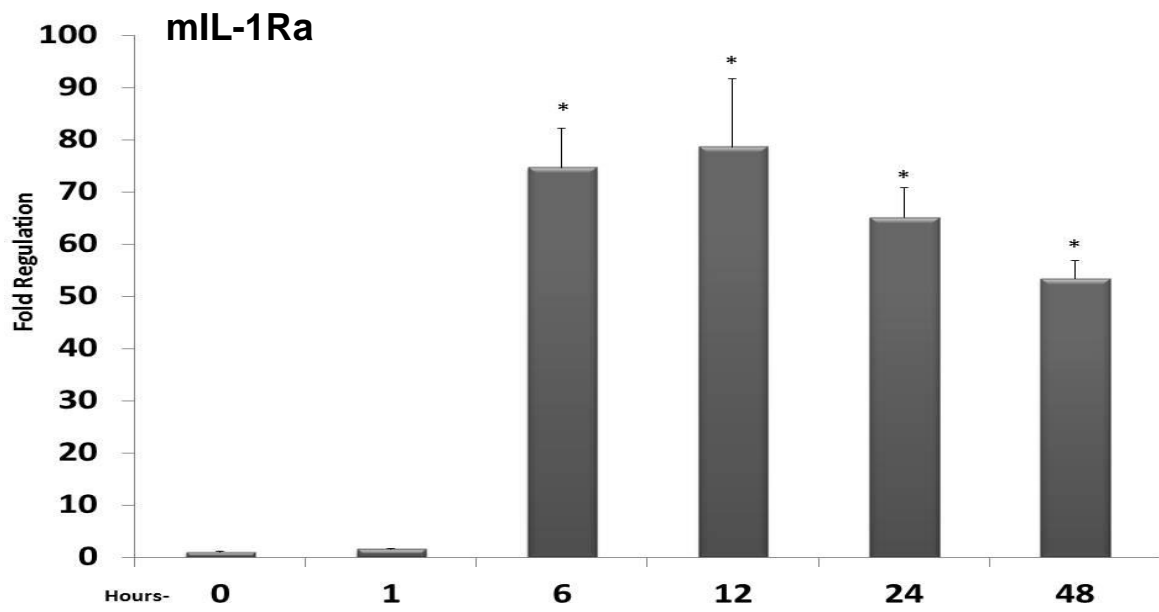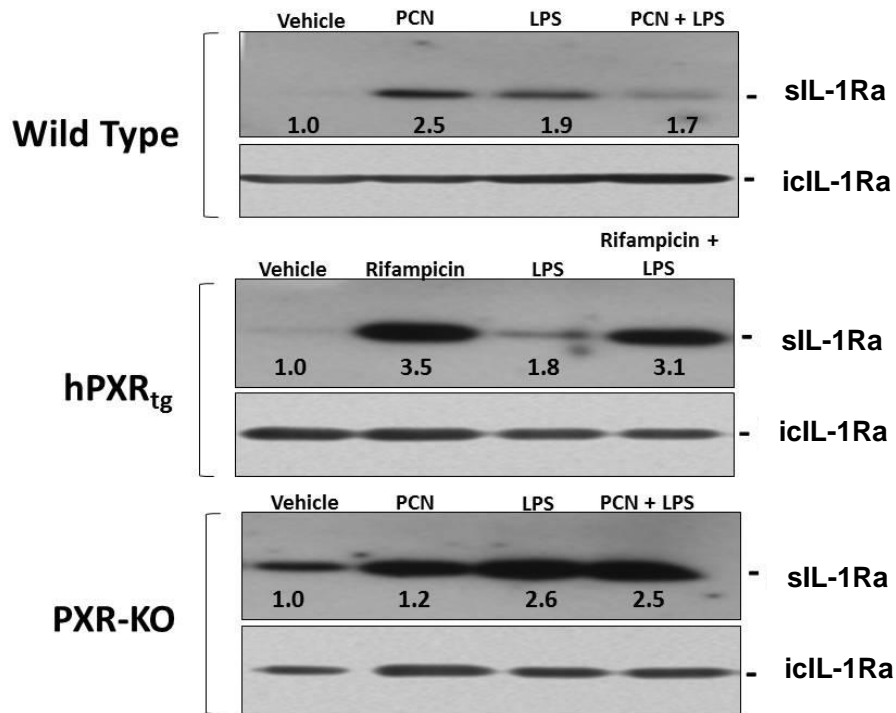**Figure 4-4**

A
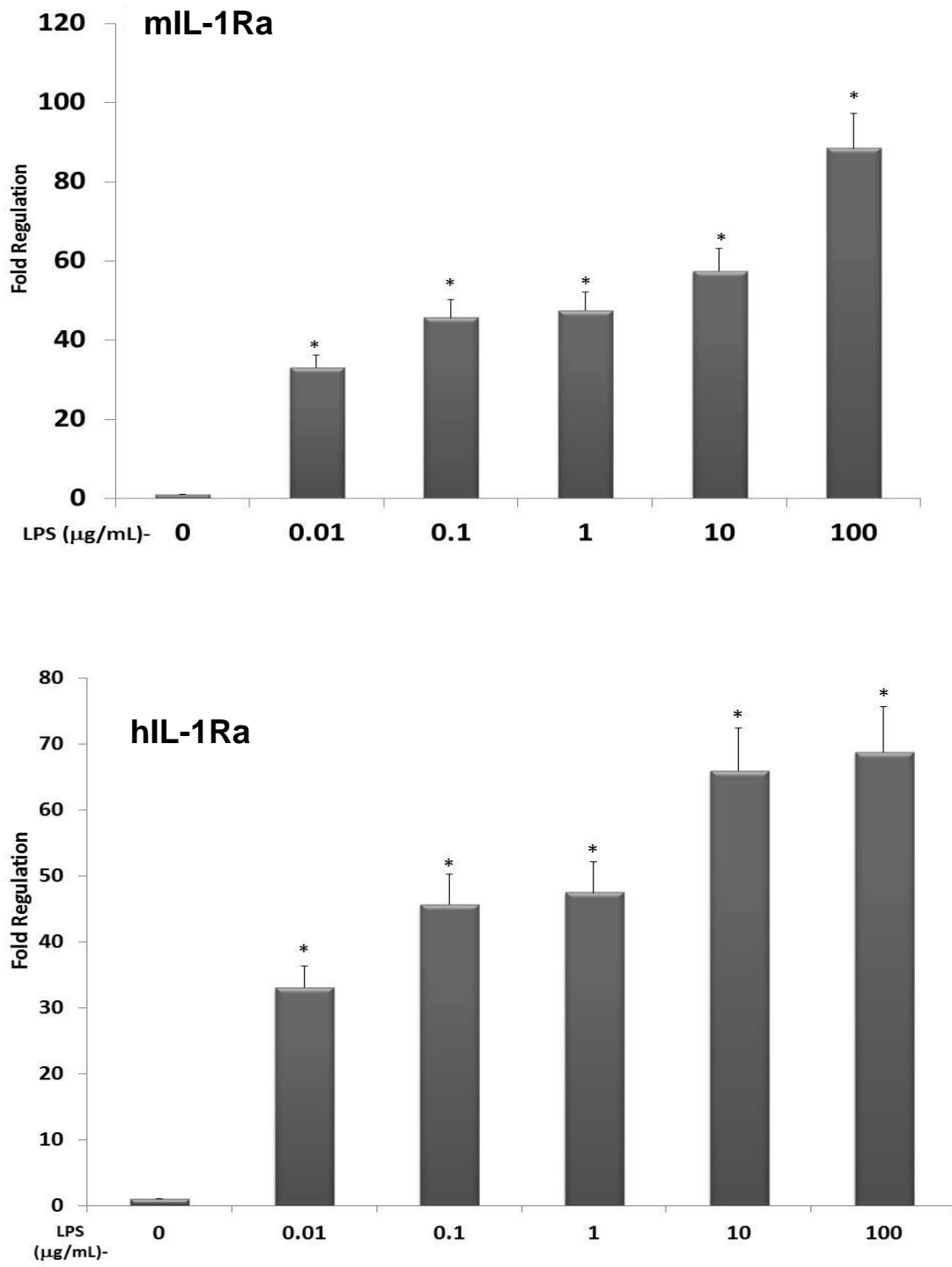
B

Figure 4-4. LPS-induced IL-1Ra Expression in Primary Hepatocytes. (A) Primary cultures of wild type mouse hepatocytes and human hepatocytes were treated with vehicle or indicated concentrations of LPS for 12 hours. Total RNA was isolated and RT-qPCR analyses were performed to determine the expression of IL-1Ra. (B) Primary cultures of wild type mouse hepatocytes and human hepatocytes were treated with vehicle or LPS (10 μg/ml) for indicated time points. Total RNA was isolated and RT-qPCR analyses were performed to detect the expression levels of IL-1Ra through time.   All data are normalized to β-actin levels and data are expressed as fold regulation compared with that observed in veh-treated wild type cells.

respectively (Fig. 4-5A).   When treated with PCN or rifampicin for 36 hours, the expression

level of sIL-1Ra in the media increased from hepatocytes of both wild type and hPXR$_{tg}$ mice.

For cells that were treated with LPS alone and cells which were pre-treated with PCN or

rifampicin, then co-treated with LPS, the level of sIL-1Ra also increased, but the induction

level was lower compared to PXR activation alone.   When hepatocytes from PXR-KO mice

were used in identical experiments, PCN had no effect on sIL-1Ra protein induction.   In

contrast, 12 hours of LPS treatment led to an enhanced induction of sIL-1Ra in PXR-KO

hepatocytes compared to PXR-positive cultures.   Additionally, co-treatment of PXR-KO

hepatocytes with PCN and LPS failed to diminish sIL-1Ra levels.   Taken together, the data

presented indicate that PXR activation has both an early negative regulatory role in the

LPS-inducible expression of key inflammatory mediators like IL-1β and IL-6, as well as a

likely positive role in regulating ligand-inducible expression of the secreted form of IL-1Ra

protein at later time points.   To more closely examine the potential positive role of PXR

activation in regulating sIL-1Ra protein levels across species, a longer time-course study

using primary human hepatocytes was conducted (Fig. 4-5B).   Treatment of human

hepatocytes with rifampicin for 48 hours produced a robust induction in sIL-1Ra levels,

whereas 24 hours of treatment with LPS produced less induction compared to rifampicin

treatment.   Treatment of human hepatocytes with rifampicin for 48 hours, followed by

co-treatment with rifampicin and LPS for an additional 24 hours produced an induction of

sIL-1Ra much higher than rifampicin treatment alone.   These data indicate that long-term

pre-activation of PXR in both rodent and human hepatocytes has a strong positive effect on
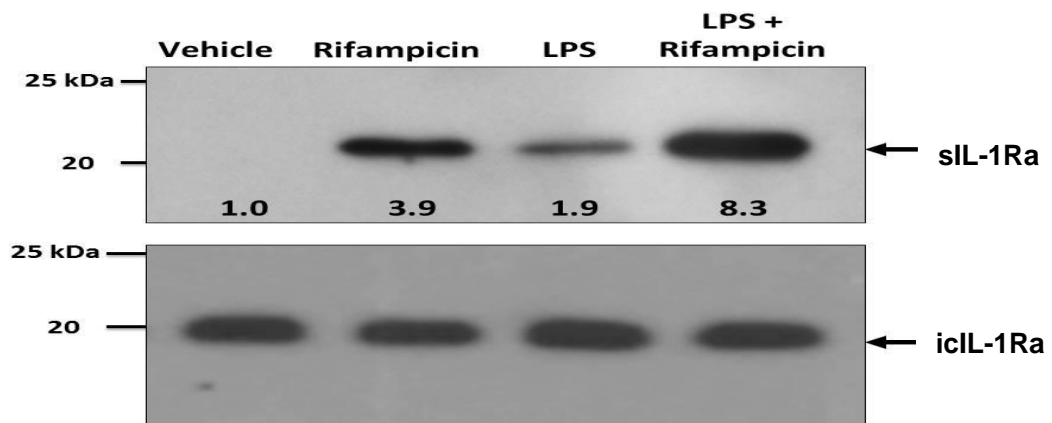
sIL-1Ra production.

Figure. 4-5

A

B

Figure 4-5. **Analysis of the Secreted Form of IL-1Ra Protein in Culture Media from Primary Hepatocytes Isolated from Wild Type, hPXR$_{tg}$, PXR-KO Mice and Human Hepatocytes.** (A) Primary mouse hepatocytes were isolated from the indicated genotype and were treated with vehicle (0.1% DMSO) or 10μM PCN for 24 hours. Cell cultures were then divided into four experimental groups and were treated for an additional 12 hours with either vehicle, PCN alone, 10μg/ml LPS alone, or PCN and LPS together. Western Blot analysis of the secreted form (sIL-Ra) and intracellular form (icIL-1Ra) of IL-1Ra was performed. (B) Primary human hepatocytes were treated with vehicle (0.1% DMSO) or 10μM Rif for 48 hours. The cultures were then divided into four experimental groups and were treated for an additional 24 hours with either vehicle, Rif alone, 10μg/ml LPS alone, or Rif and LPS together. Western Blot analysis of the secreted form (sIL-Ra) and intracellular form (icIL-1Ra) of IL-1Ra was performed. Western Blot images were quantitated by densitometric scanning of the X-ray film with the UVP Biodoc-It 220 image analysis system and 1D Gel Analysis Software. The numbers represent densitometric image intensity of sIL-1Ra divided by image intensity of icIL-1Ra.

4.3.2 SUMOylation Regulates the Inhibitory Role of PXR on Inflammation

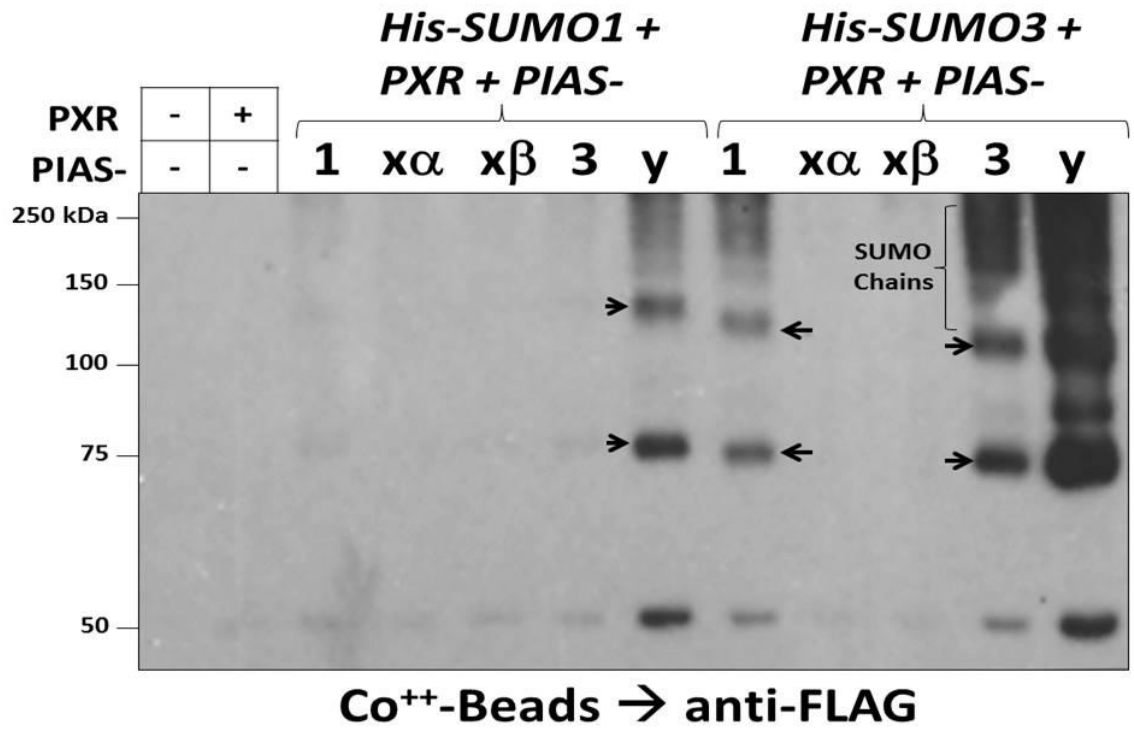**The SUMOylation and De-SUMOylation of PXR in Cell-based SUMOylation Assay.**

Hepa1-6 cell, a mouse hepatoma cell line, was used in our study. This cell line expresses endogenous SUMOs at high levels, and is highly permissive for SUMOylation compared to other commonly used cell lines like HeLa and CV-1 cells (data not shown). Moreover, Hepa1-6 cells do not express endogenous PXR (data not shown). PIAS proteins are a family of well-characterized SUMO E3 ligases. There are five unique PIAS proteins (PIAS1, PIASxα, PIAS3, PIASxβ, and PIASy). Each PIAS family member exhibits distinct but sometimes overlapping SUMO E3 ligase enzymatic activity towards substrates. The specific SUMO E3 ligase that promotes SUMO-modification of PXR is currently unknown. To determine which PIAS family member(s) could function as E3 ligase(s) towards PXR, Hepa1-6 cells were co-transfected with expression vectors encoding FLAG-tagged PXR together with His-tagged SUMO1 or SUMO3 and an additional expression vector encoding a specific member of the PIAS family. As shown in Fig.4-6A, in the presence of PIASy, the SUMO(1)ylation of PXR was robustly promoted at two sites. When SUMO3 was used in the same assay, more robust SUMO-chain formation was observed with PIASy. PIAS1 and PIAS3 also promoted SUMO(3)ylation of PXR. In general, the signal for SUMO(3)ylation was stronger than SUMO(1)ylation, indicating PXR was preferentially SUMOylated by SUMO3 in Hepa1-6 cells. This is consistent with our previous publication on human PXR (26). Since PIASy enhanced PXR SUMOylation, for the following study, it was used to promote PXR SUMOylation.

There is increasing recognition that regulation of SUMO modification also occurs at the level of de-SUMOylation. Similar to E3 ligases, the specific SENP(s) that remove SUMO modification from PXR are currently unknown. After the identification of E3 ligase for PXR SUMOylation, we next sought to identify the SENP family member(s) that de-SUMOylates PXR using a variation of our cell-based SUMOylation assay. Expression vectors encoding His-tagged SUMOs, PIASy and FLAG-tagged PXR were co-transfected into Hepa1-6 cells together with selected SENPs as indicated in the figure. Where available, the catalytically-deficient mutant form of each SENP was used as negative controls. As shown in Fig. 4-6 (B), SENP2 completely abolished SUMOylation of PXR, whereas the catalytically deficient form of SENP2 was ineffective. While expression of SENP1 and SENP6 promoted de-SUMOylation of PXR to some extent, the removal was incomplete. It is noteworthy that the 52 kDa immunoreactive band that corresponds to non-modified PXR increases in direct proportion to the level of SUMO-modification. These data suggest that PIASy-mediated SUMOylation of PXR may inhibit its proteasome-mediated degradation. Identical experiments using SUMO3 indicate that SENP1, SENP3, and SENP6 de-SUMOylating enzymes selectively remove SUMO-chains, while SENP2 is the most effective at removing all SUMO moieties from PXR.

Activation of PXR Suppresses the Expression of Inflammatory Response Genes in Hepa1-6 Cells. It has been shown that activation of PXR repressed the inflammatory response in hepatocytes. However, it was difficult to manipulate primary hepatocytes, thus Hepa1-6 cells were used to further the study. mPXR expression vector was transfected into Hepa1-6 cells before the treatment with PXR ligand and inflammatory stimuli. As shown in

Figure 4-6

A

B

His-SUMO1 + PIASy + PXR

His-SUMO3 + PIASy + PXR
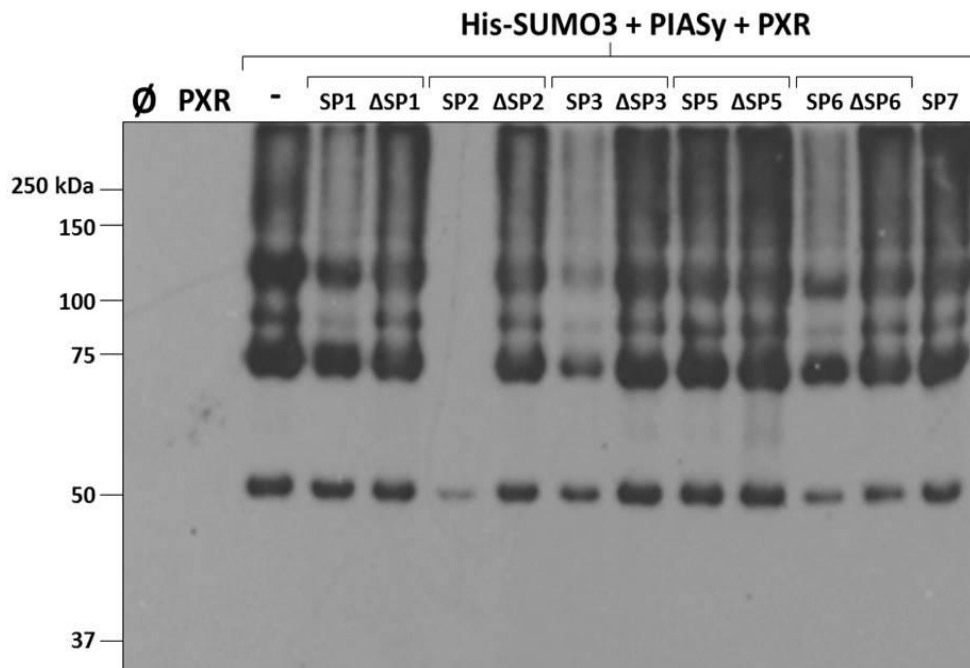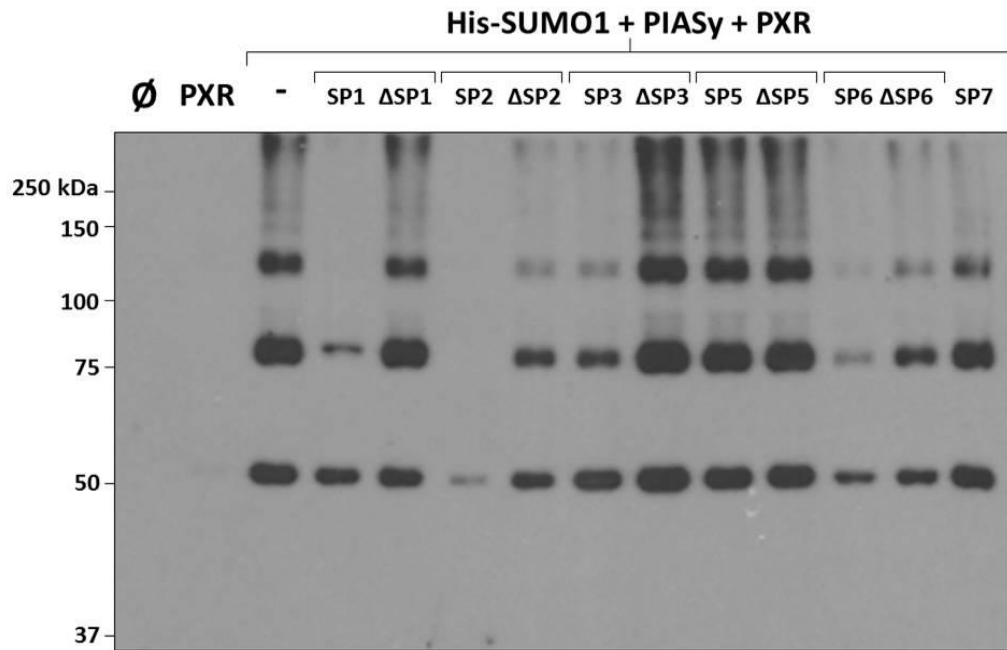
Co⁺⁺-Beads → anti-FLAG

Figure 4-6. Characterization of PXR SUMOylation and De-SUMOylation. (A) In order to facilitate SUMOylation of PXR, mammalian expression vectors encoding (His)$_6$-tagged SUMO1/3, PIASs and FLAG-tagged mPXR were co-transfected into Hepa1-6 cells as indicated. 48 hours post-transfection, cells were harvested in denaturing buffer and SUMOylated proteins were purified with cobalt-linked agarose beads. Captured proteins were subjected to SDS–PAGE and subsequent western blot analysis using an anti-FLAG antibody (PA1-984B, Thermo Scientific). (B) To determine the ability of SENPs to de-SUMOylate mPXR, expression vectors encoding (His)$_6$-tagged SUMO1/3, PIASy, FLAG-tagged mPXR and various SENPs and corresponding mutants were transfected into Hepa1-6 cells as indicated. 48 hours post-transfection, cells were harvested in denaturing buffer and SUMOylated proteins were purified with cobalt-linked agarose beads. Captured proteins were subjected to SDS–PAGE and subsequent western blot analysis using an anti-FLAG antibody (PA1-984B, Thermo Scientific).

Fig.4-7(A), introduction of exogenous PXR restored drug-inducible Cyp3a11 gene expression, indicating that exogenous PXR could be activated in Hepa1-6 cells.   In accordance with the results in WT hepatocytes, LPS also dampened PCN-induced Cyp3a11 expression in Hepa1-6 cells.   After the confirmation that exogenous PXR functioned well in Hepa1-6 cells, we next sought to further examine the effect of PXR on LPS-induced inflammation.   In non-transfected cells, LPS treatment significantly induced the expression of both Cox-2 and IL-6 and PCN treatment had no repressive effect on LPS-induced gene expression, as shown in Fig. 4-7(B).   After PXR was introduced into the Hepa1-6 cells, PCN treatment profoundly repressed Cox-2 and IL-6 gene expression.   Moreover, LPS-inducible Cox-2 and IL-6 expression was abolished in the presence of PXR, regardless of treatment with LPS or a combination LPS and PCN (As shown in Fig. 4-7B).   These results indicate that in Hepa1-6 cells, exogenous PXR is a strong repressor of the inflammatory response.

Expression of De-SUMOylation Enzymes Impairs the Ability of PXR to Suppress the Expression of Inflammatory Response Genes.   Previously, we demonstrated that SENP2 was the major enzyme that de-SUMOylates PXR and SENP6 could remove SUMO chains from PXR.   To further confirm the effect of SUMOylation on PXR-mediated repression of inflammatory mediators, expression vectors for PXR and SENP2 or SENP6 were co-transfected into Hepa1-6 cells.   As shown in Fig. 4-8, the expression of exogenous PXR alone still abolished LPS-induced expression of Cox-2 and IL-6.   However, in the presence of SENP2 PXR no longer suppressed LPS-induced expression of Cox-2 and IL-6, suggesting SUMOylation was indispensable for PXR-mediated repression.   Interestingly,
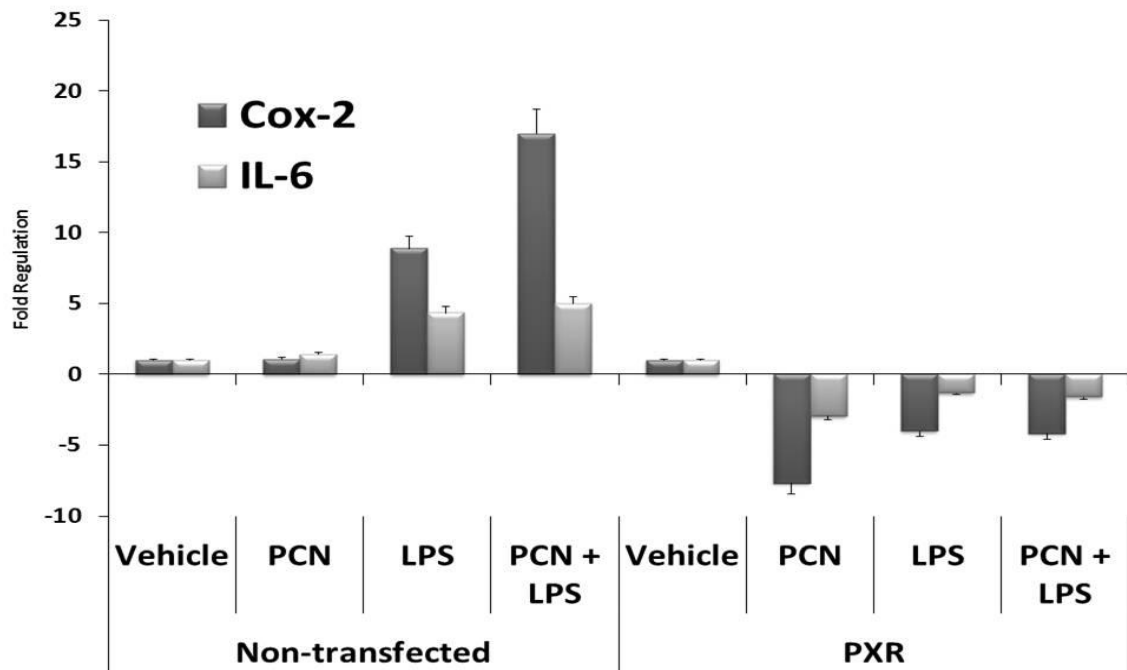
Figure 4-7

A
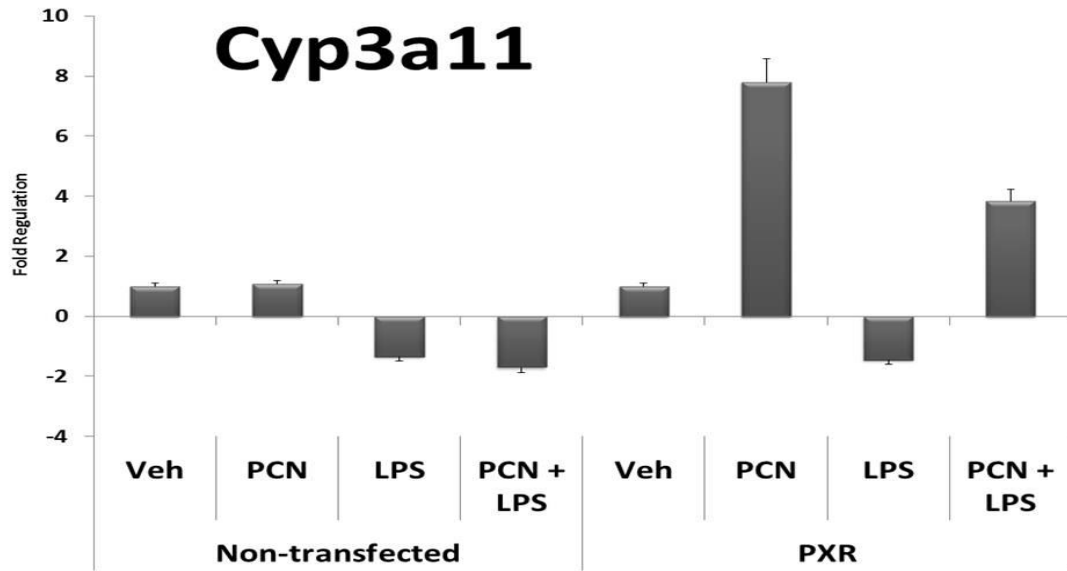
B

Figure 4-7.   Activation of Exogenous PXR Represses LPS-inducible Inflammatory Response Genes in Hepa1-6 Cells.   Hepa1-6 cells were transfected with expression vector encoding FLAG-tagged mPXR.   24 hours post-transfection, cells were treated with 10 μM PCN for 36 hours followed by an additional 12 hours treatment with or without LPS (10 μg/ml).   Total RNA was isolated, and the relative mRNA expression levels of (A) Cyp3a11 and (B) Cox-2 and IL-6 were determined by RT-qPCR.   The expression level of each gene in vehicle treated non-transfected cells was set as 1.   All data are normalized to β-actin levels and displayed as fold regulation.

Figure 4-8
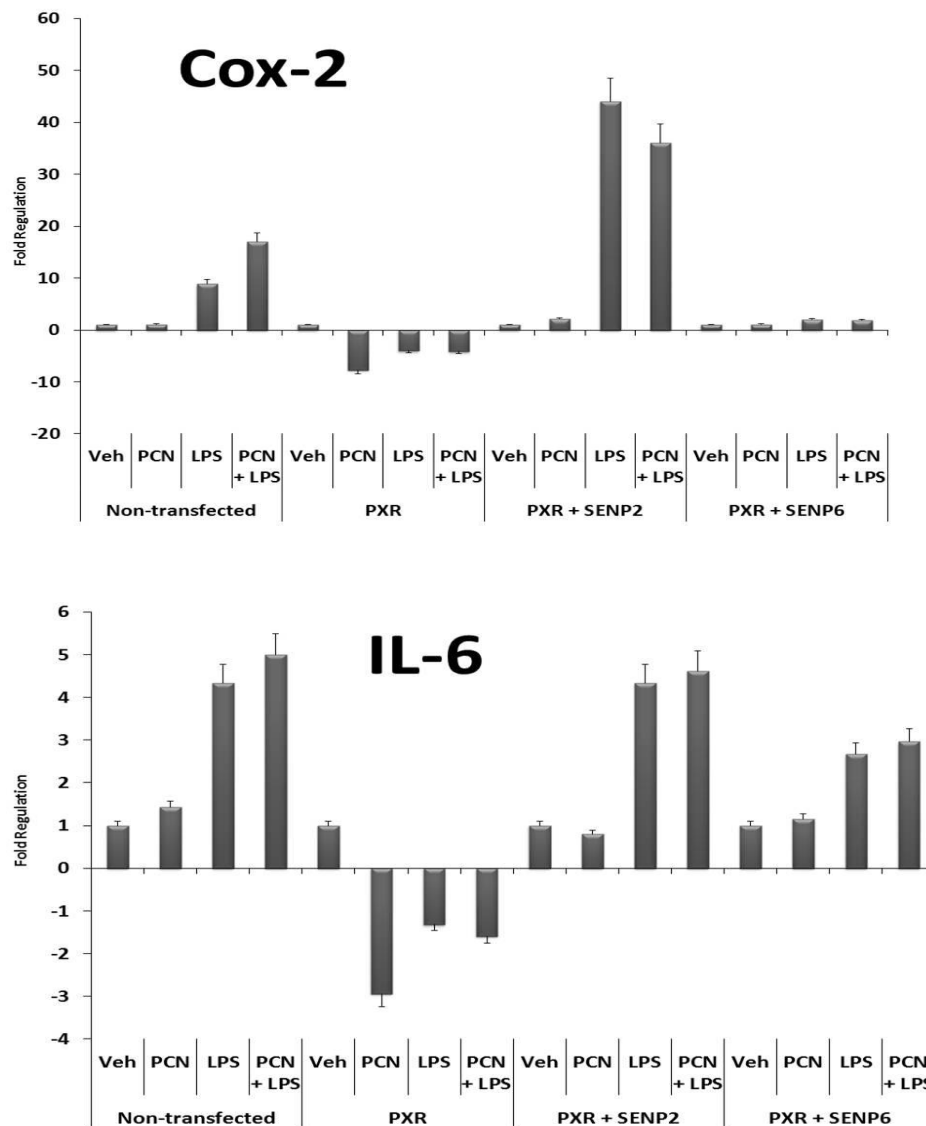
Figure 4-8. The Effects of SENPs on the Expression of PXR-suppressed Inflammatory Response Genes. Hepa1-6 cells were transfected with expression vectors encoding mPXR and SENP2 or SENP6. 24 hours post-transfection, cells were treated with 10 μM PCN for 36 hours followed by an additional 12 hours treatment with or without LPS (10 μg/ml). Total RNA was isolated, and the relative mRNA expression levels of IL6 and Cox-2 were determined by RT-qPCR. The expression level of each gene in vehicle treated non-transfected cells was set as 1. All data are normalized to β-actin levels and are expressed as fold regulation.

while the expression of SENP6 partially restored LPS-induced IL-6 expression, it had no effect on LPS-induced Cox-2 expression in cells expressing exogenous PXR (Fig. 4-8). These data raise the question of whether SUMO chains are specifically required for the repression of certain genes and whether the mechanism(s) underlying PXR-mediated repression differs between various genes.

4.4 Discussion

Many NR ligands, like glucocorticoids are widely used as anti-inflammatory drugs. Numerous NRs have been shown to regulate both innate and adaptive immune systems through different mechanisms. For instance, GR interferes with the assembly of co-activator complexes and the transcription elongation factor b complex, which are required for NF-κB activation, thus inhibiting the expression of NF-κB target genes (32-34). Moreover, GR can modulate MAPK (Mitogen-activated Protein Kinase) signaling to inhibit the expression of AP-1 induced inflammatory genes (35-37). SUMOylated LXRs, LRH1 and PPARγ can prevent the removal of corepressor complexes from the promoter of inflammatory response genes (25, 38, 39). It is well-accepted that activation of PXR suppresses inflammatory response. However, activation of NF-κB also represses PXR target gene expression. This mutual repression is known as transrepression (29). Several possible mechanisms have been proposed for the inflammation-induced suppression of drug metabolism and clearance. Inflammatory stimuli have been shown to reduce the mRNA levels of PXR and disrupt the association between PXR and DNA (40, 41). Though the plausible mechanism for inflammation-mediated PXR target gene repression has been proposed, the underlying mechanism(s) for PXR-mediated repression on inflammatory response still remains vague. Our data indicate that SUMOylation of PXR is involved in PXR-mediated repression.

Over-expression of SENP2, the major enzyme that de-SUMOylates PXR, abolishes the ability of PXR to repress LPS-induced expression of Cox-2 and IL-6. However, expression of SENP6, which removes SUMO3 chains from PXR, has a different effect on PXR-repressed Cox-2 and IL-6 expression. It is plausible that the underlying mechanism for PXR mediated repression is gene or promoter specific. One important step to further confirm SUMOylation's role is to identify SUMOylation site(s) in PXR and generate SUMOylation deficient PXR mutants. However, current research in our laboratory using site-directed mutagenesis indicates that SUMOylation can occur on multiple lysine sites within PXR and it is highly possible that when the major SUMOylation sites are mutated, SUMOylation can migrate to other lysine sites. Another interesting aspect is to see whether SUMOylated PXR shares the same mechanism with LXR, LRH1 and PPARγ to interfere with the removal of corepressor complexes from the promoters of inflammatory response genes. Since the activity of PXR is affected by multiple signaling pathways, further studies are required to see how different signaling pathways affect PXR SUMOylation and the ability of PXR to repress inflammatory response; whether post-translational modifications of PXR-interacting proteins contribute to PXR mediated repression on inflammation; and also, whether disease states alter the ability of PXR to repress inflammation.

PXR has evolved to protect the body from toxic xenobiotic insults. Its function is evolutionarily conserved, but there are some species differences. For instance, PCN is a strong rodent PXR agonist, but it has little effect on human PXR. Moreover, activation of the cyclic AMP-dependent protein kinase signaling pathway increases PXR-mediated gene activation in mouse hepatocytes, whereas the same signaling pathway represses PXR-mediated gene activation in rat and human hepatocytes (42). Thus, it is important to look at PXR activity in different species. In this chapter, we mainly focused on mouse PXR. However, SUMOylation of PXR is conserved between mouse and human PXRs, so are the

potential SUMOylation sites.   Our previous work showed that hPXR repressed inflammatory responses in human immortalized cell lines (26).   In rat, treatment with PXR ligands has also been shown to ameliorate intestinal inflammation, indicating the anti-inflammatory function of PXR is evolutionally conserved through different species (43, 44).

The data presented here indicate that PXR activation has both an early negative regulatory role in LPS-inducible expression of key inflammatory mediators like IL-1β and IL-6, as well as a likely positive role in regulating ligand-inducible expression of the secreted form of IL-1Ra protein at later time points.   The working model for the feedback inhibition and resolution of the inflammatory response in hepatocytes through time is depicted in Figure 4-9.   Based on the observations discussed above, we propose that following injury or infection, low stoichiometric amounts of SUMO-modified PXR transcriptionally suppresses pro-inflammatory mediators like IL-1β and IL-6, while the remainder of PXR protein is likely ubiquitinated and subsequently degraded by the 26S proteasome in a signal dependent manner. Consistent with the hypothesis, significant lower levels of PXR protein are detected in endotoxin-treated mice (45).   As the inflammation ensues through time, newly synthesized PXR protein becomes available for up-regulating ligand-dependent expression of novel or alternative PXR-target genes including IL-1Ra, possibly through cryptic or low-affinity PXR-response elements.   Moreover, peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1α), a strong PXR co-activator, is known to control the expression of IL-1Ra in the liver (46).   In this way, PXR activation gains anti-inflammatory function and plays an active role in the resolution of inflammatory response.

Understanding the underlying mechanism of how PXR converts from a positive regulator of xenobiotics clearance to a repressor of inflammatory response will lead to the development of new treatments to control inflammation.   Further understanding of PXR together with other NRs will definitely lead to improvements on current drug therapies and also the development of novel drugs.
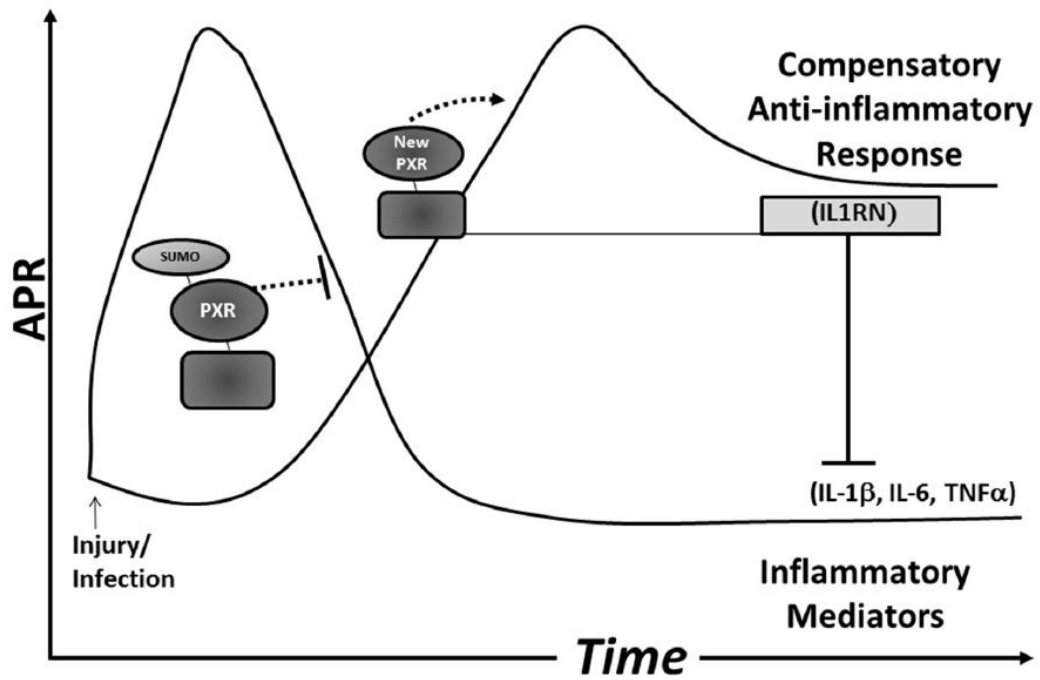
Figure 4-9

Figure 4-9. Model of the Mechanism of PXR-mediated Interaction with the

Inflammatory Response in Hepatocytes.

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Chapter 5: Future Directions

5.1 Identification of Post-Translational Modifications Sites within the PXR Protein.

The identification of PXR modification sites provides information for crosstalk between different post-translational modifications and is an important step towards understanding the biological roles of different modifications. The identification of PXR modification sites has always been a research interest in our laboratory. While the mutation of acceptor lysine to arginine supposes to inhibit modifications like ubiquitination and SUMOylation, our data indicate that neither SUMOylation nor ubiquitination can be totally knocked out by single lysine mutation. The data indicate that there are multiple modification sites. Or the modification can be migratory. In other words, when the major modification site(s) is unavailable, the modification can occur at sites that are not modified under normal conditions. Beside biochemical methods such as site-directed mutagenesis, another commonly used approach to identify modification sites is mass spectrometry. *In vitro* SUMOylation and *in vitro* ubiquitination assays are wildly used for this purpose (1, 2). Because the stoichiometry of PXR SUMOylation is extremely low, several improvements were employed to increase PXR SUMOylation in *in vitro* assays, including escalating protein amounts, incorporating identified SUMO E3 ligase and increasing the reaction time. After the improvements, SUMOylated PXR bands can be visualized in Coomassie Blue stained gels (As shown in Fig.5-1). All these efforts laid the foundation for mass spectrometry-based identification. Here we only used SUMOylation as an example. Similar approach can be applied to other modifications. The identification of PXR modification sites will facilitate our understanding
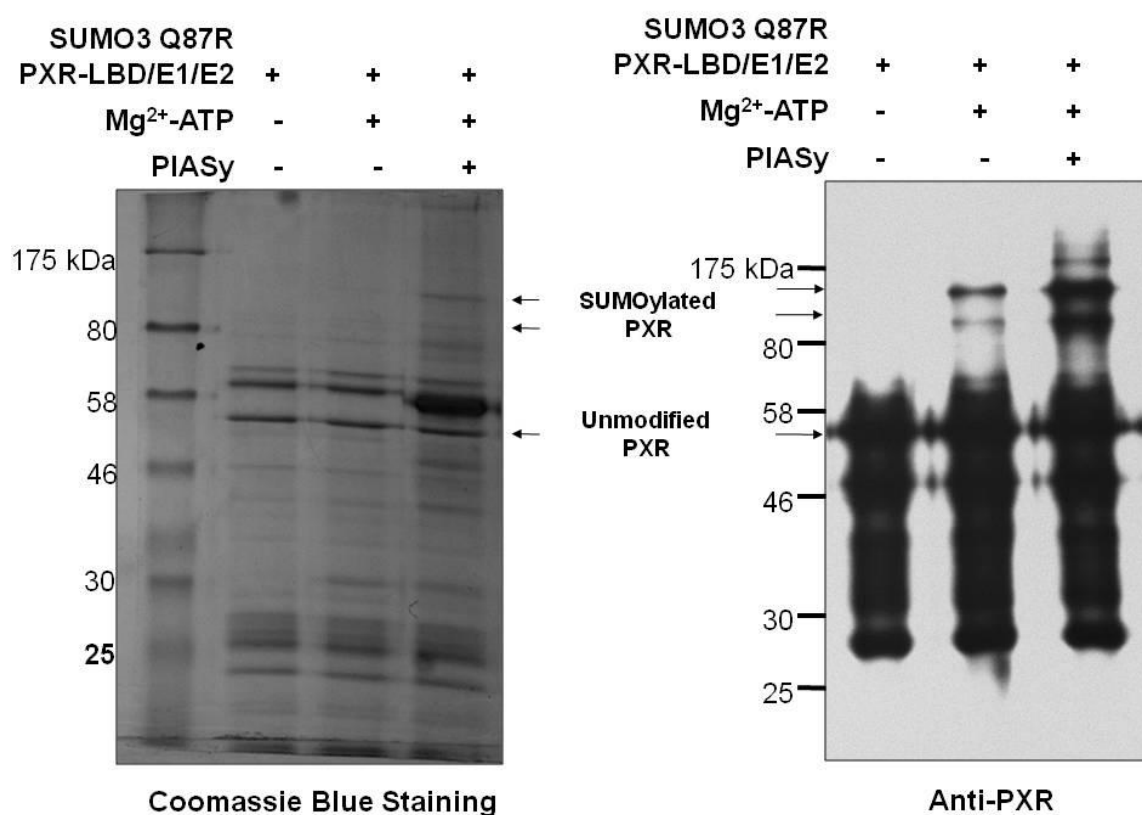
Figure 5-1

Figure 5-1. *In Vitro* **SUMOylation Assay for GST-hPXR-LBD.** 20 μl reactions containing 8μl of purified GST-hPXR-LBD, 2 μl of E1 enzyme (Enzo Life Sciences), 2 μl of E2 enzyme (Enzo Life Sciences), with or without 2 μl of PIASy together with 1μl purified SUMO3 Q87R were incubated at 37 ℃ in the presence or absence of Mg^{2+}-ATP for 3 hours. After the addition of 20μl of 2 × SDS-PAGE gel loading buffer, 5μl sample was subjected to western blot analysis with antibodies that recognize PXR (sc-48340, Santa Cruz) and the rest of the sample was subjected to Coomassie Blue staining.

of the biological functions and physiological roles of different modifications.

5.2 The Development of Research Models for PXR

The species difference of PXR is well-recognized, which hinders the translation of data generated from animal models to clinical outcomes. Currently, for PXR studies, the most relevant model is primary human hepatocytes. However, the limited source of human liver remains to be an issue, and regardless hepatocyte cultures cannot fully represent the *in vivo* situation. Using murine hepatocytes, Zellmer *et al.* showed that after isolation, hepatocyte cultures showed major alterations in gene expression compared to the *in vivo* situation (3). The mRNA levels of various CYP450 enzymes are differentially expressed over time in hepatocyte cultures. For the first 1–2 days, time-dependent decreases in the mRNA levels are observed for all major CYP450 genes. Then the expression levels of some enzymes like CYP3A4 and CYP2D6 can recover to a certain degree, while others including CYP1A2 and CYP2E1 do not recover (4). To overcome the differences between primary cultures of hepatocytes and the *in vivo* situation, increasing modifications have been made to the culture system, like the collagen sandwich cultures, which can preserve the polarized hepatocytes morphology and facilitate long-term culture. Many commercially available novel hepatocyte culture models are developed. For example, the hepatocytes are cultured together with non-parenchymal cells. Using the Transwell approach and the hanging drop strategy, 3D structure is allowed to develop in the culture system (5).

As developments have been made to *in vitro* cell culture systems, the progress in mice model development is also remarkable. Transgenic mice expressing human PXR are

generated (6, 7). The PXR of a transgenic mouse can be selectively activated by human

PXR ligand rifampicin, suggesting the model overcomes some aspect of species specificity.

The generation of double transgenic mice expressing both human PXR and human CYP3A4

provides another way to overcome the limitations of transgenic mouse models (8). The

development of transgenic mouse models allows us to study the function of PXR in a whole

animal system to reveal the physiologic functions of PXR. Even though both *in vitro* and *in

vivo* models have improved significantly, they still need to be interpreted with caution when

experimental data are extrapolated to humans.

5.3 Regulation of PXR in Patho- and Physiological Conditions

As discussed previously, PXR plays regulatory roles in many patho- and physiological

conditions. Activation of PXR is suggested to be beneficial in the treatment of diverse

diseases, like cholestasis and inflammatory bowel diseases (9, 10). Many widly used

medications later turned out to be PXR ligands and their efficacy is at least partially through

the activation of PXR, as in the case of rifaxinmin (11). Previously, activation of PXR has

mainly been suggested to treat various metabolic diseases. However, recent studies indicate

that the therapeutic value of PXR is wider than formerly thought. One example is in the

treatment of Alzheimer's disease (AD). P-glycoprotein (Pgp), also known as multidrug

resistance protein 1 (MDR1), is a well-characterized target gene of PXR. Pgp is expressed

in many tissues including intestinal epithelium, hepatocytes, and in the capillary

endothelial cells comprising the blood–brain barrier. Amyloid β-peptide (Aβ) accumulation

and inflammation among many other factors are the central components of AD

pathophysiology. Pgp regulates the clearance of Aβ from the brain into the blood (12). Using transgenic human APP (amyloid precursor protein)-over-expressing mice, Hartz *et al.* showed that activation of PXR by PCN restored Pgp expression and transport activity in brain capillaries and significantly reduced brain Aβ levels (13). Moreover, the anti-inflammation property of PXR also contributes to the management of AD. Since PXR activators manifest favorable effects on AD management, PXR is implicated as an emerging therapeutic target for AD. While the primary function ascribed to PXR is the homeostatic control of steroids, bile acids, and xenobiotics, the wide distribution of PXR indicates that it might be involved in many other pathways. The identification of novel roles of PXR in different patho- and physiological conditions, novel ligands and novel target genes is an important aspect of PXR research. The broadening of our understanding of PXR may lead to novel therapeutic strategies, just like the case in AD treatment.

On the other hand, activation of PXR is not without risks. In the transgenic mice where PXR is constitutively activated, growth retardation, hepatomegaly and histological liver toxicity are observed, suggesting that sustained activation of PXR can be harmful (7). Furthermore, it is obvious that activation of PXR is involved in adverse drug-drug interactions, especially in patients who take multiple medications. If one drug is a PXR ligand, the activation of PXR can lead to accelerated metabolism and clearance of other co-administered medications. For medications with narrow therapeutic indices, like digoxin and warfarin, alterations in metabolism and clearance can often lead to life-threatening consequences. Studies of PXR have deepened our understanding of adverse drug-drug interactions and allowed us to predict the potential of drug-drug interactions in the early stage

of drug development. Nowadays, drug candidates are routinely tested for their ability to activate PXR in pharmaceutical companies. The compounds that activate PXR will be withdrawn or modified to minimize their PXR activating property, which can prevent late-stage clinical failures and minimize the costs. In addition to adverse drug-drug interactions, PXR activation may also correspond to the underlying mechanism for some drug-induced pathological conditions, like impaired immune responses and hepatic steatosis.

Recent studies indicate that post-translational modifications also regulate the activities of PXR, representing new modes of PXR-mediated gene regulation. Data generated in our laboratory indicate that the presence of corepressor proteins like SMRT and NCoR can increase the SUMOylation level of PXR (As shown in Fig. 5-2). Whether other PXR interacting proteins also regulate post-translational modifications of PXR, and ascertaining if these regulations occur in response to metabolic, pathogenic, and xenobiotic stress remain interesting issues to be explored. Furthermore, the identification of molecules that directly modulate PXR post-translational modification status without activating PXR is another important aspect of PXR research. Similar research has been done with PPARγ. It has been shown that blocking CDK5-mediated PPARγ phosphorylation by non-agonistic binding produces anti-diabetic effects without some of the side effects of thiazolidinediones (14). Targeting the post-translational modifications, instead of direct PXR agonism, may be a better way to get therapeutic effects and avoid potential side effects like adverse drug-drug interactions.

The inhibition of PXR activities also has gained much attention, not only to avoid

Figure 5-2

PXR	-	+	+	+	+
His-S3	-	-	+	+	+
Ubc 9	-	-	+	+	+
PIASy	-	-	+	+	+
SMRT	-	-	-	+	-
NCoR	-	-	-	-	+

175 kD —
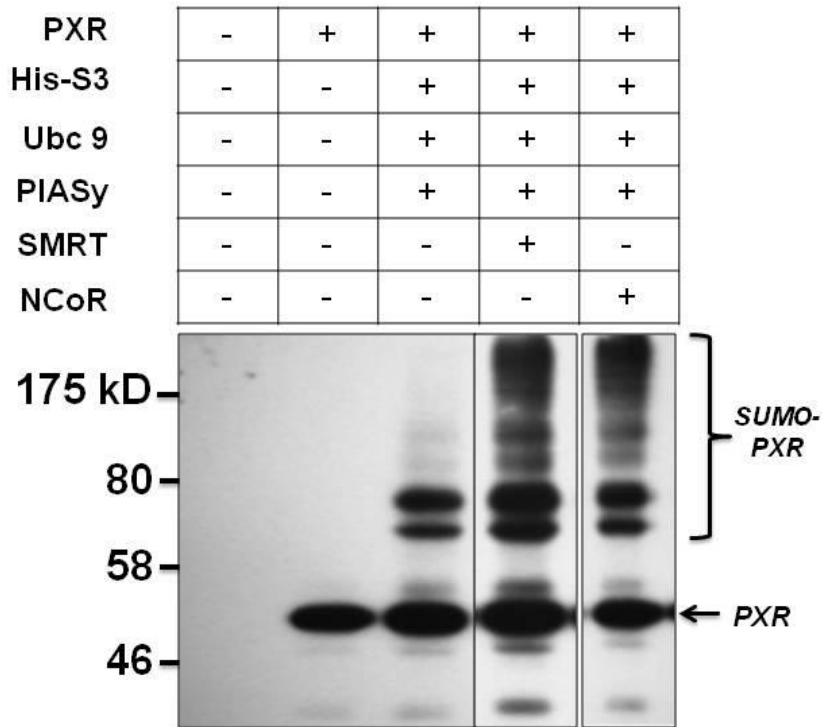80 —
58 —
46 —

SUMO-PXR

← PXR

Figure 5-2.   Corepressor Proteins Increase PXR SUMOylation.   Expression vectors encoding PXR, His-tagged SUMO3, Ubc9, PIASy, SMRT and NCoR were transfected into HeLa cells as indicated in the figure.   48 hours post-transfection, cells were harvested in denaturing buffer and SUMOylated proteins were purified with nickel-linked agarose beads. Captured proteins were subjected to SDS–PAGE and subsequent western blot analysis using an anti-PXR antibody (sc-48340, Santa Cruz).

drug-drug interactions, but also to prevent drug resistance and tumor growth in cancer patients. Furthermore, the inhibition of PXR may represent a novel management for steatosis treatment. However, compared to the numerous PXR agonists that have been detected, the discovery for PXR antagonists has just begun. Selective and non-toxic PXR antagonists are of great therapeutic potential.

Over one-and-a-half decades after its discovery, our knowledge on PXR has expanded from an orphan receptor to a well-characterized xeno- and endobiotic sensor with great therapeutic potentials. Besides its role in maintaining cellular homeostasis, PXR also serves as an attractive target for the development of pharmacologic modulators for managing many metabolic and non-metabolic diseases. The continued study of PXR will aid the development of novel, safe and effective therapeutic strategies, and help prevent drug-drug and disease-drug interactions in patients.

5.4 References

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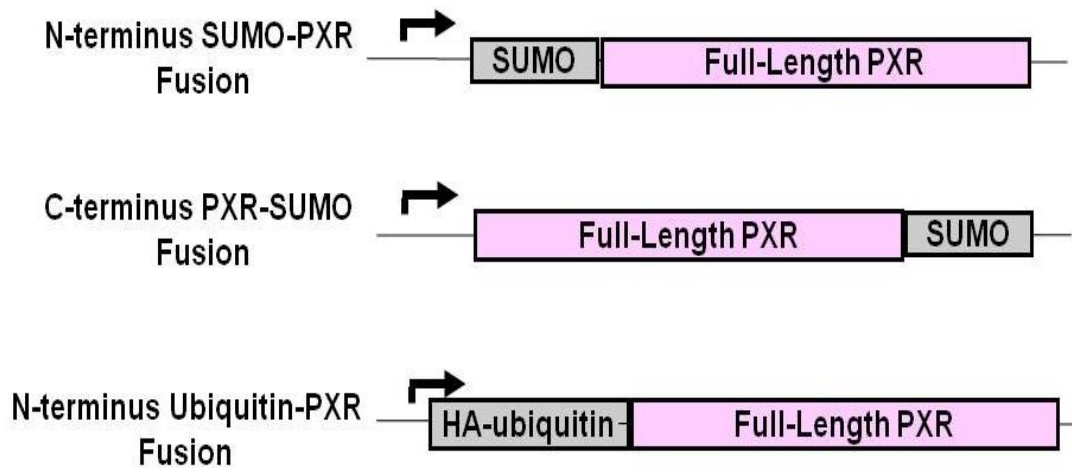# Appendix 1: The Generation and Characterization of PXR-SUMO/PXR-Ub Fusion Proteins

It is reported that linear SUMO/Ub-fusion proteins behave similarly to SUMO and Ub conjugates and can be a useful tool to study SUMOylation and ubquitination (1-3). While SUMO and ubiquitin modifications are highly dynamic and reversible, the generation of fusion protein can provide a form of protein that is constitutively attached to SUMO or ubiquitin. To study the effects of SUMOylation and ubiquitination on PXR, PXR SUMO fusion proteins were generated by incorporating the SUMO at both the N terminus and C terminus of PXR in different PXR expressing vectors. PXR ubiquitin fusion proteins were generated by incorporating the ubiquitin at the N terminus of PXR (Illustrated in Fig. S1-1A). The expression of the linear fusion proteins were confirmed using both PXR and SUMO/Ub antibodies, as shown in Fig. S1-1B-D. After confirmation of the expression of fusion proteins, the transactivation ability of these novel constructs were examined. Analysis of the XREM-Luc reporter gene assays showed that the fusion proteins could still be activated by the prototypical PXR ligand- rifampicin. However, the fusion of SUMO or ubiquitin significantly reduced the transcriptional activity of PXR (As shown in Fig.S1-2).

To test the hypothesis that SUMOylated PXR represses the inflammatory response, the SUMO fusion PXR construct was used in the NF-κB-Luc reporter assays. As shown in Fig.S1-3, in the reporter alone group, treatment of cultured HeLa cells with hTNFα produced an approximate 12-fold increase in NF-κB reporter gene activity. While the expression of hPXR effectively repressed TNFα-mediated NF-κB reporter gene activity, the SUMO fusion PXR had no further repression effect compared to the wild type PXR. Since the NF-κB-Luc reporter used in the experiment only contains NF-κB-response elements, which is a highly
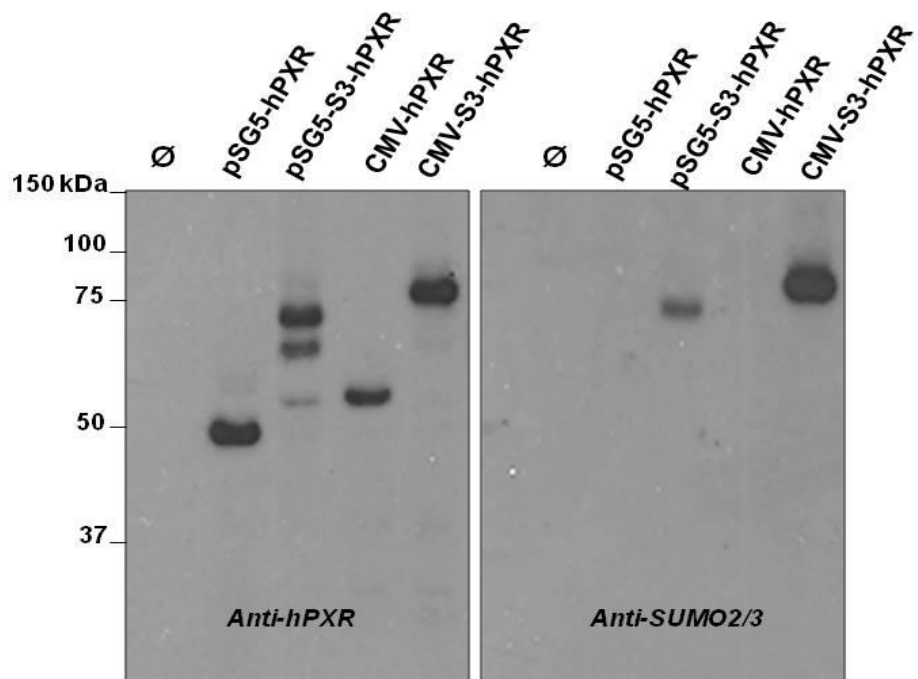
simplified model to study inflammatory response, it is possible that the repression of PXR on inflammatory response requires other transcription factors and their response elements.   The data presented in Chapter 4 also suggest that the ability of PXR to repress inflammatory response genes is gene- or promoter- specific.   Future experiments introducing both wild type and PXR SUMO fusion proteins into PXR-KO hepatocytes and testing the expression of specific inflammatory response genes will be useful to further test our hypothesis.

Figure S1-1

A

N-terminus SUMO-PXR Fusion

→ [SUMO] [Full-Length PXR]

C-terminus PXR-SUMO Fusion

→ [Full-Length PXR] [SUMO]

N-terminus Ubiquitin-PXR Fusion

→ [HA-ubiquitin] [Full-Length PXR]

B

Anti-hPXR *Anti-SUMO2/3*

C
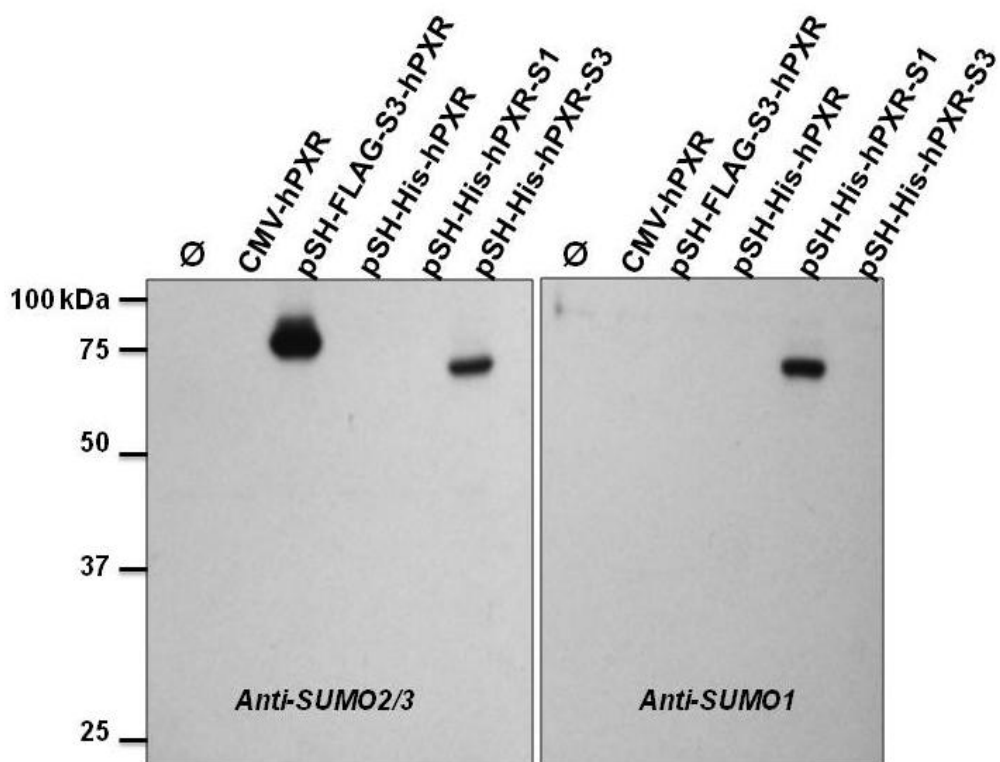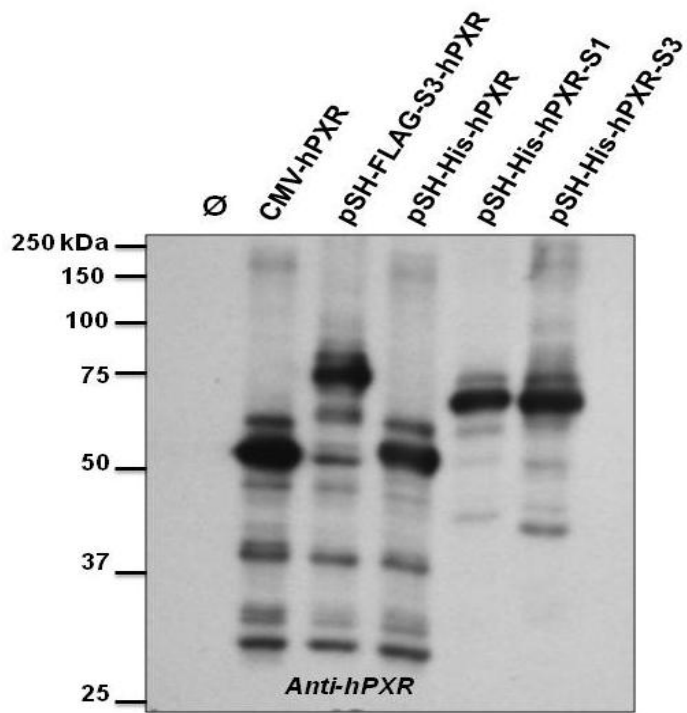
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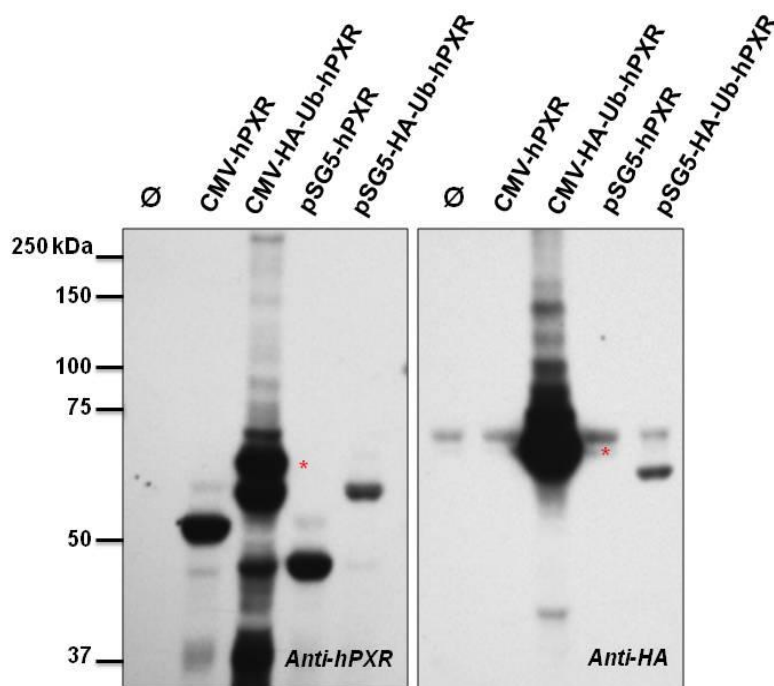
Figure S1-1. The Expression of SUMO/Ub PXR Fusion Proteins. (A) Schematic

representation of PXR SUMO and ubiquitin fusions.   For N-terminus fusions, SUMOs and

ubiquitin, which lack one of the two glycines at the C terminus and the stop codon, were

fused in-frame to full-length PXR.   For C-terminus fusions, the stop codon of full length

PXR was removed before its fusion to SUMOs, which lack one of the two glycines at the C

terminus of mature SUMOs.   The removal of one of the two glycines at the C terminus of

mature SUMOs and ubiquitin prevents the fusion proteins from being processed by

SUMO/De-SUMO and ubiquitination enzymes.   (B) Expression of N-terminus SUMO

fusion proteins.   Hepa1-6 cells were transfected with plasmids encoding PXR or

SUMO3-PXR fusion proteins as indicated in the figure.   Whole cell lysates were resolved by

using 10% SDS-PAGE.   The blots were probed for both PXR (Santa Cruz) and SUMO3
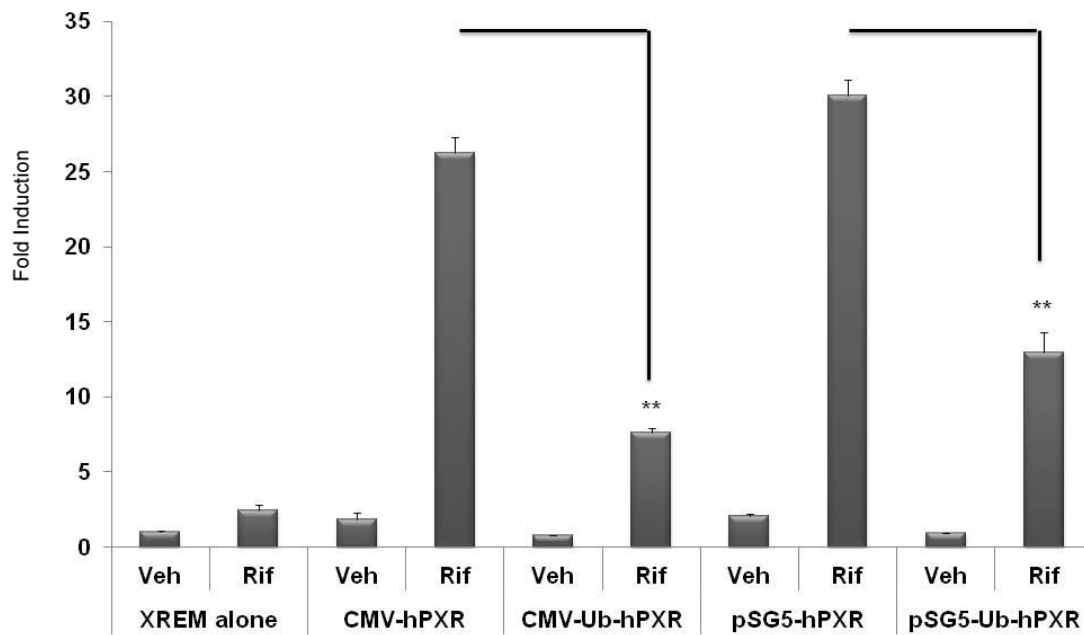
(Cell Signaling) immunoreactivity. (C) Expression of C-terminus PXR-SUMO fusion proteins. Hepa1-6 cells were transfected with plasmids encoding PXR or PXR-SUMO fusion proteins as indicated in the figure. Whole cell lysates were resolved by using 10% SDS-PAGE. The blots were probed for both PXR (Santa Cruz) and SUMO1/3 (Cell Signaling) immunoreactivity. (D) Expression of N-terminus Ub-PXR fusion proteins. Hepa1-6 cells were transfected with plasmids encoding PXR or Ub-PXR fusion proteins as indicated in the figure. Whole cell lysates were resolved by using 10% SDS-PAGE. The blots were probed for both PXR (Santa Cruz) and HA (Covance) immunoreactivity.

Figure S1-2

A
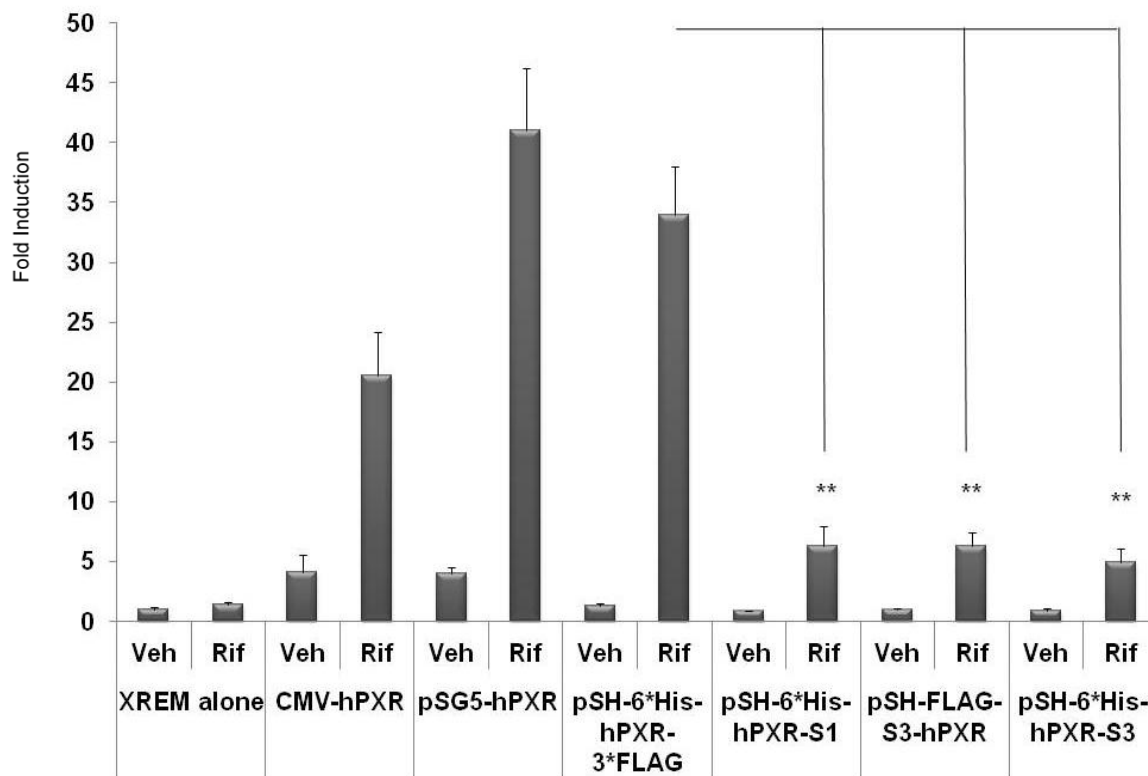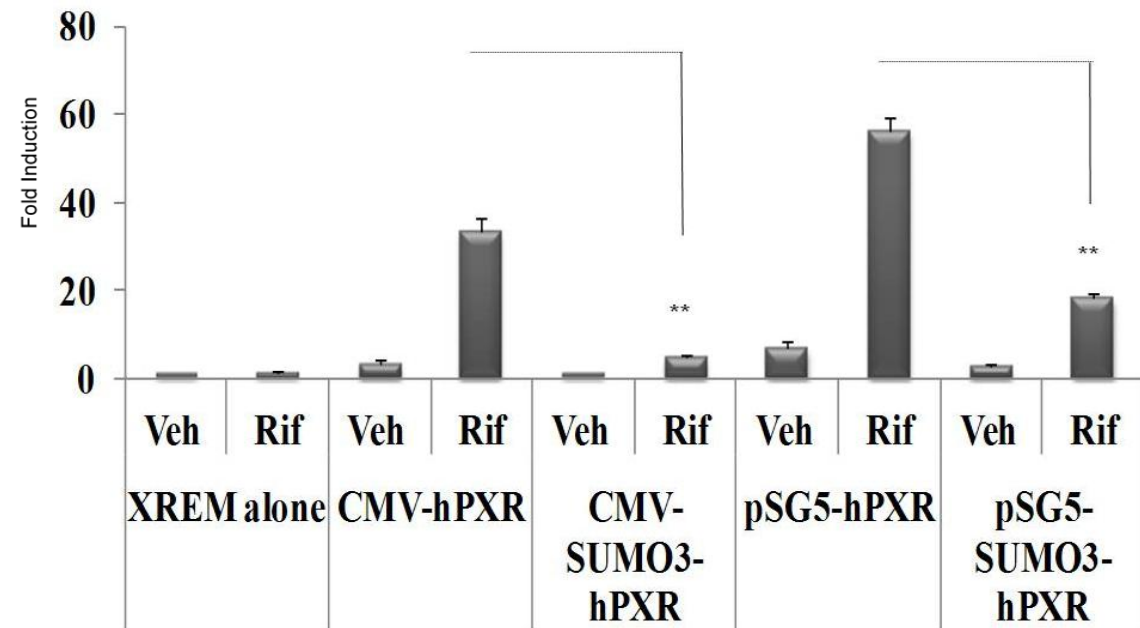
B

. **Figure S1-2.** **Linear SUMO/Ub Fusion Decreases Transactivation of PXR in XREM-Luc Reporter Assay**. (A) CV-1 cells were transfected with the XREM-luciferase reporter gene together with plasmids encoding PXR or PXR SUMO fusion proteins as indicated in the figure. 24 hours after transfection, cells were treated with vehicle (0.1% DMSO) or 10 μM rifampicin for another 24 hours. Luciferase activity was determined by using standard luciferase assay system (Promega). The results are reported as fold-induction ± S.E.M. and are normalized to β-galactosidase activity (**=p<0.001). (B) CV-1 cells were transfected with the XREM-luciferase reporter gene together with plasmids encoding PXR or Ubiquitin PXR fusion proteins as indicated in the figure. 24 hours after transfection, cells were treated with vehicle (0.1% DMSO) or 10 μM rifampicin for another 24 hours. Luciferase activity was determined by using standard luciferase assay system (Promega). The results are reported as fold-induction ± S.E.M. and are normalized to β-galactosidase activity. (**=p<0.001)
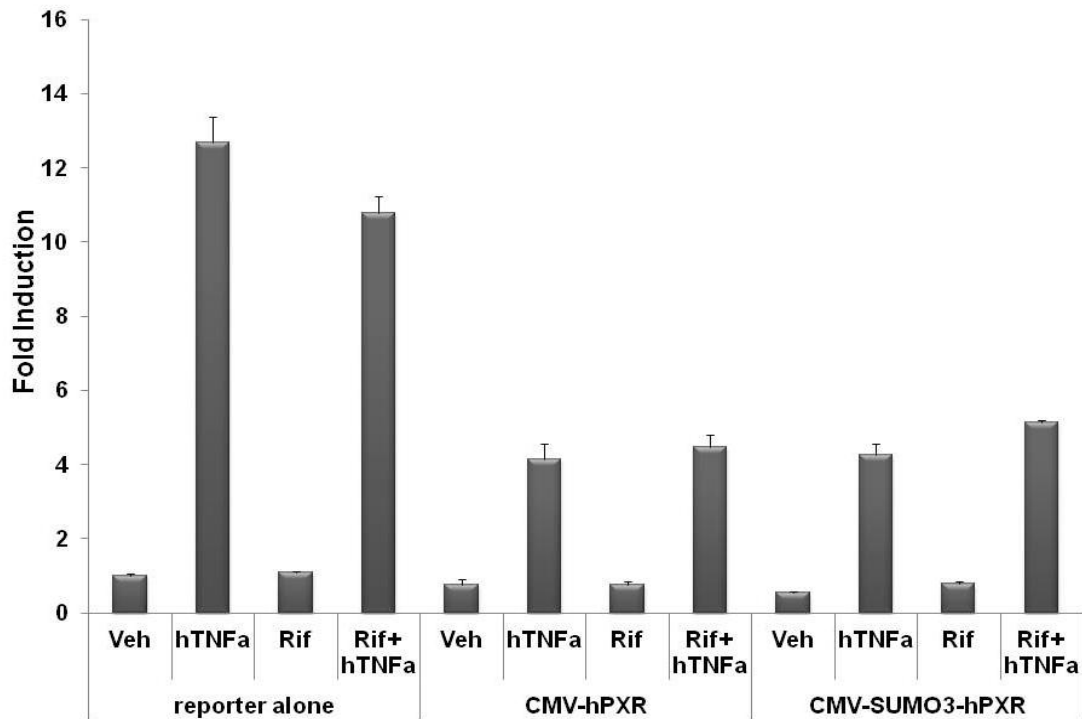
Figure S1-3. PXR SUMO Fusion Protein Has No Further Effect on the Transrepression of PXR in NF-κB-Luc Reporter Assay. HeLa cells were transfected with the NF-κB-luciferase reporter gene together with plasmids encoding PXR or SUMO3-PXR fusion protein as indicated in the figure. After transfection, cells were pre-treated with 10 μM rifampicin for 24 hours and then co-treated with hTNFα (10 ng/ml) for another 6 hours before luciferase activity was determined by using standard luciferase assay system (Promega). The results are reported as fold-induction ± S.E.M. and are normalized to β-galactosidase activity.

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Appendix 2: The Effects of PXR Activation on MAPK Activities

The mitogen-activated protein kinases (MAPKs) are a family of signal transduction proteins that can convert extracellular signals, such as the presence of mitogens, inflammatory cytokines, and growth factors, to the intracellular pathways through a series of phosphoryltaion events.   Three subfamilies of MAPKs are well-characterized: ERKs (extracellular signal-regulated protein kinases), JNKs (c-jun N-terminal kinases) and the p38 (1).   Activation of PXR has been shown to elicit p38 phosphorylation and lead to cell migration.   The induction is mediated by GADD45β.   GADD45β is known to activate p38 signaling pathway through direct interaction with MTK1 (also known as MEKK4) (2, 3). PXR can directly activate GADD45β gene expression by binding to GADD45β promoter and then activate the p38 signaling pathway (4).   The notion that PXR activation might affect MAPK activities prompted us to further the study using wild type mouse hepatocytes.
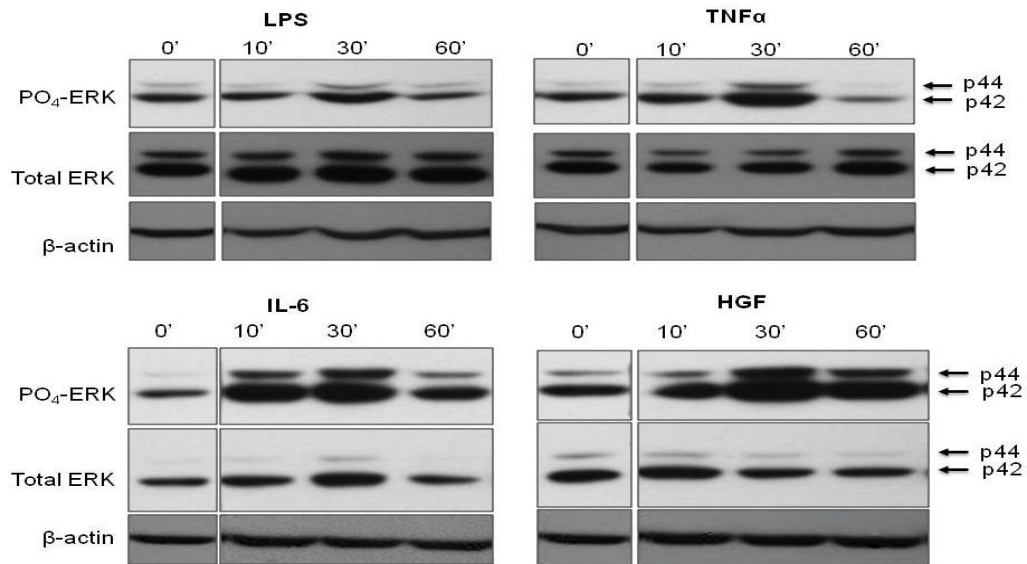
After isolation, primary cultures of mouse hepatocytes were allowed to attach overnight. Firstly, time-dependent analysis was conducted.   Several well-known activators of MAPKs, including LPS, TNFα, IL-6, and HGF (hepatocyte growth factor), were used in the experiment.   Wild type mouse hepatocytes were treated for different time points (10 min, 30min, and 60 min) before whole cell lysates were harvested and subjected to Western Blot analysis.   As shown in Fig. S2-1, for all the treatments, phosphorylation of MAPKs showed robust induction at 30 min, and the phosphorylation levels decreased at 60 min.   The data indicate that phosphorylation of MAPKs is a quick and transient process.   Since in our experimental settings, phosphorylation levels of MAPKs showed great induction at 30 min,

for the following experiments, 30 min was chosen for MAPKs stimuli treatment.

Next we sought to determine how PXR activation alters the phosphorylation events of MAPKs. Wild type mouse hepatocytes were pre-treated with vehicle or 10 μM PCN for 48 hours. Cell cultures were then divided into four experimental groups and were treated for an additional 30 min with either vehicle, PCN alone, MAPKs stimuli alone, or PCN and MAPKs stimuli together. Then whole cell lysates were harvested and subjected to Western Blot analysis. As shown in Fig.S2-2 (A), treatment of PCN alone had no significant effect on the phosphorylation of ERK and JNK. However, activation of PXR led to increased phosphorylation of p38. This is in accordance with the previous publication (4). When compared the co-treatment groups to the stimuli alone treatment groups, the pre-activation of PXR led to slight increase at the phophorylation levels of MAPKs, as shown in Fig. S2-2(B-D).
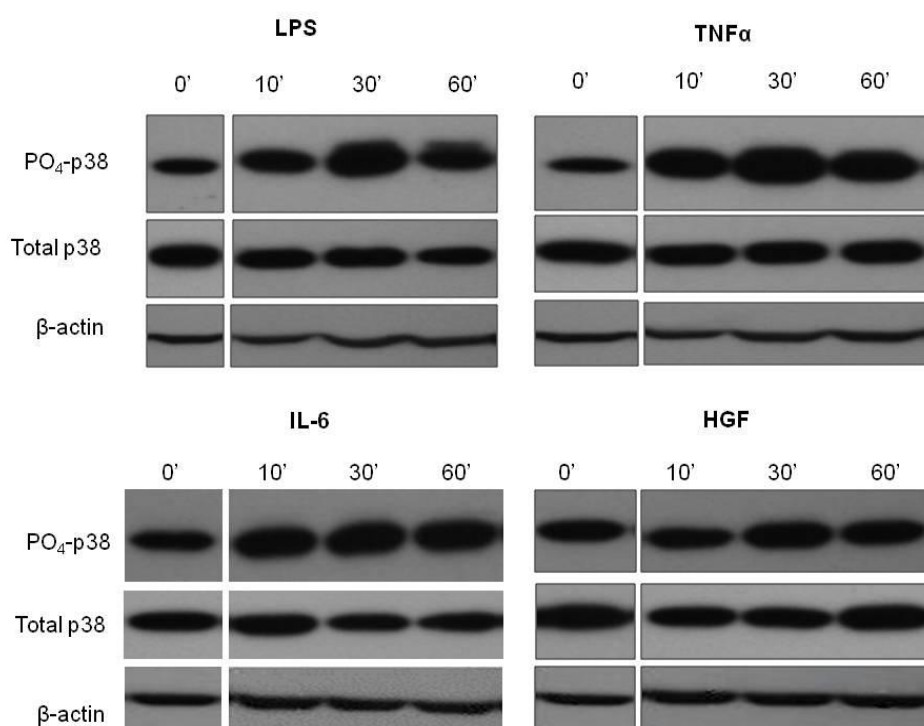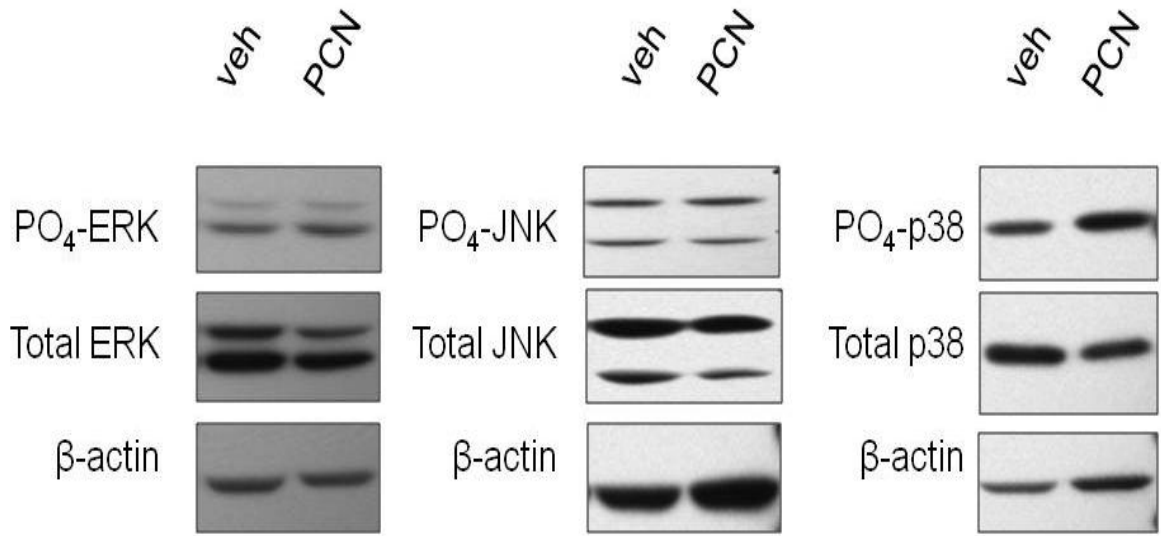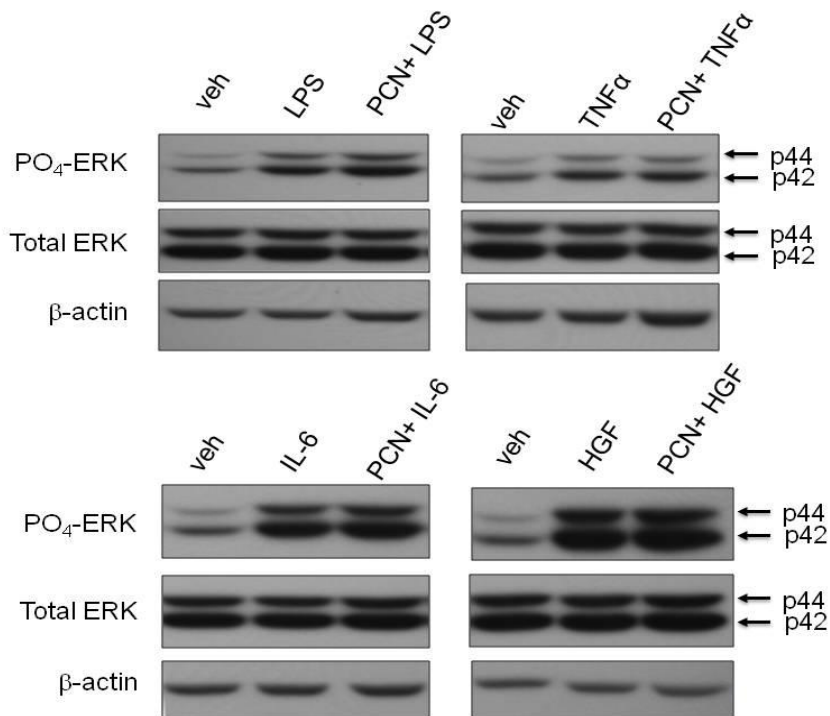
Figure S2-1

A

B
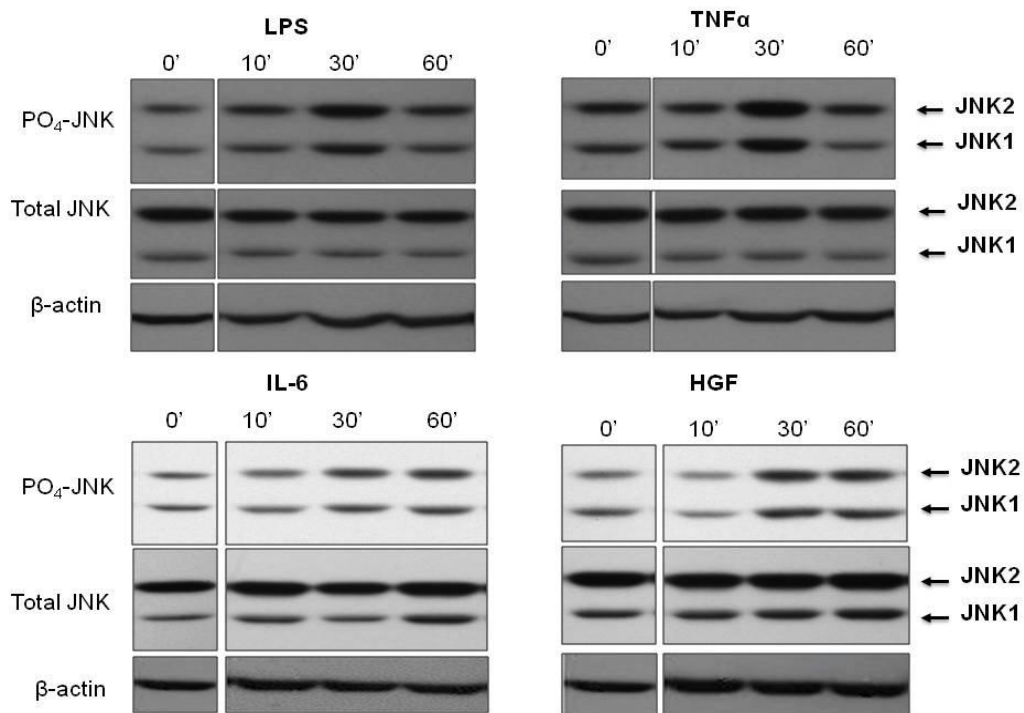
C

Figure S2-1. The Effects of Different Stimuli on the Phosphorylation Status of MAPKs.

Cultures of primary mouse hepatocytes were isolated from wild type mice.   After overnight

attachment, hepatocytes were treated with LPS (20 μg/ml), TNFα (20 ng/ml), IL-6 (40 ng/ml),

and HGF (20 ng/ml) for indicated time points.   Whole cell lysates were harvested and

subjected to SDS–PAGE and subsequent western blot analysis using (A) ERK/PO$_4$-ERK, (B)

JNK/PO$_4$-JNK, and (C) p38/PO$_4$-p38 antibodies (Cell Signaling).   β-actin was used as
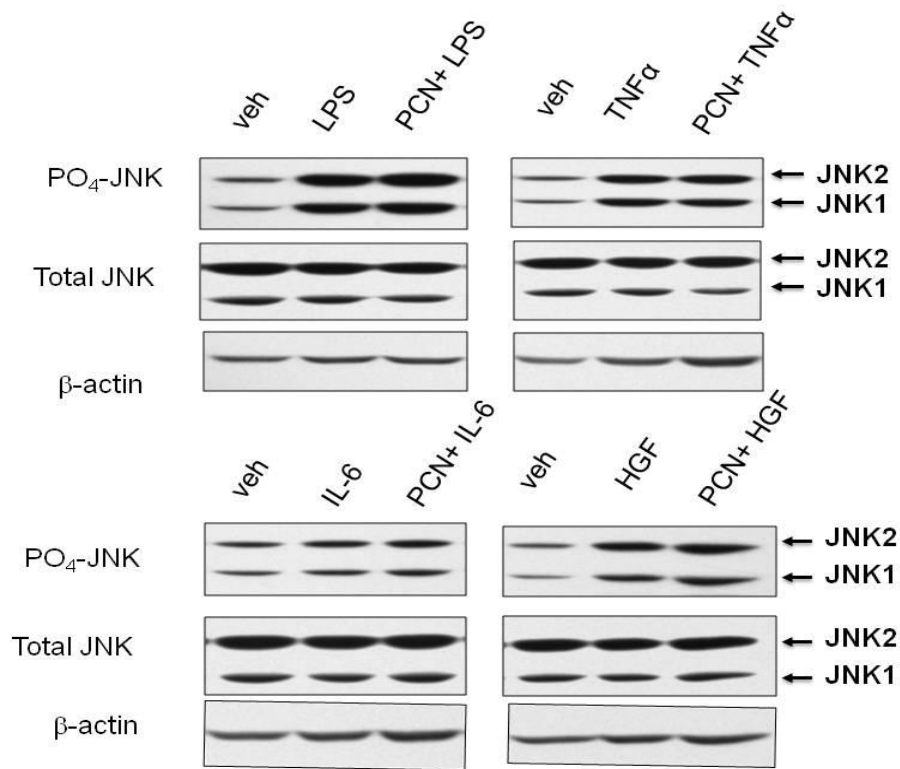
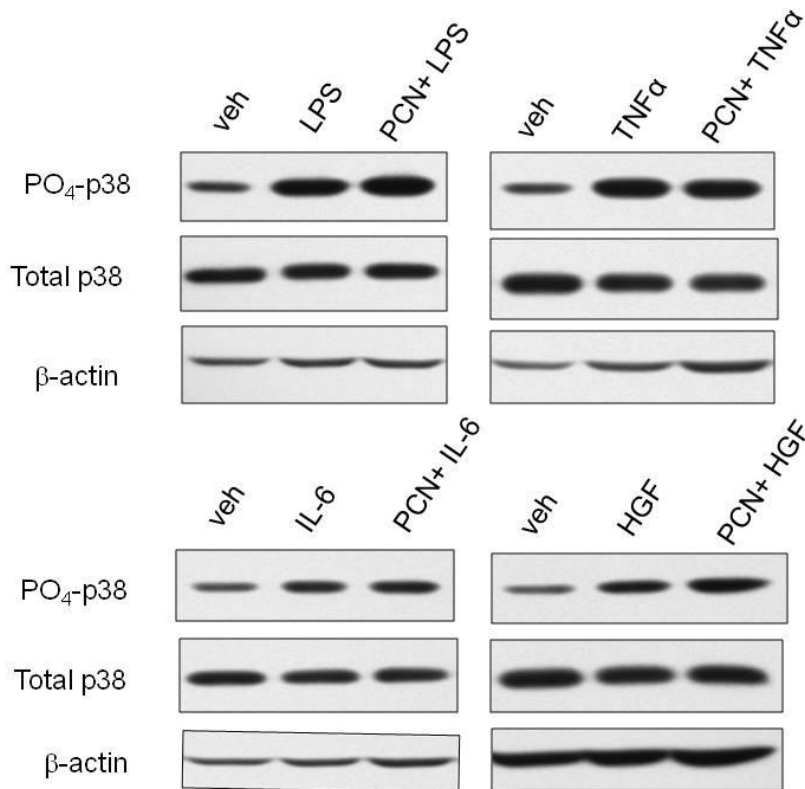loading control.

Figure S2-2

C

D

Figure S2-2. The Effects of PXR Activation on the Phosphorylation Status of MAPKs.

Cultures of primary mouse hepatocytes were isolated from wild type mice. (A) Hepatocytes were treated with vehicle (0.1% DMSO) or 10 μM PCN for 48 hours before whole cell lysates were harvested and subjected to SDS–PAGE and subsequent western blot analysis using ERK/PO$_4$-ERK, JNK/PO$_4$-JNK, and p38/PO$_4$-p38 antibodies (Cell Signaling). β-actin was used as loading control. (B-D) Wild type hepatocytes were treated with vehicle (0.1% DMSO) or 10 μM PCN for 48 hours before the treatment with LPS (20 μg/ml), TNFα (20 ng/ml), IL-6 (40 ng/ml), and HGF (20 ng/ml) for 30 min. Whole cell lysates were harvested and subjected to SDS–PAGE and subsequent western blot analysis using (B) ERK/PO$_4$-ERK, (C) JNK/PO$_4$-JNK, and (D) p38/PO$_4$-p38 antibodies (Cell Signaling). β-actin was used as loading control.

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