Pregnane X Receptor SUMOylation and De-SUMOylation

By

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Abstract

The pregnane x receptor (PXR, NR1I2) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors. PXR is activated by numerous lipophilic compounds, a variety of drugs and drug metabolites in clinical use. It regulates xenobiotic-inducible cytochrome P450 (CYP) expression in the liver and intestine which are major organs for xenobiotic biotransformation. While PXR has been identified as the positive regulator of many drug metabolizing enzymes (DMEs) and membrane transporter proteins, the effect of certain PXR activators is to repress the inflammatory response. Although it has been known for 40 years now that the PXR activator rifampicin inhibits immunological responses in liver cells, the mechanisms remain poorly understood. Previous results indicate that modification of PXR by small ubiquitin-related modifier (SUMO) likely contributes to this phenomenon. We hypothesize that PXR is SUMOylated to transrepress the inflammatory response genes in liver. Here, this thesis examines PXR SUMOylation/de-SUMOylation reaction mechanisms using *in vitro* and cell-based methods. Bacterial expression and purification systems were used in conjunction with *in vitro* SUMOylation reactions to further analyze PXR SUMOylation by both SUMO-1 and SUMO-3. The data reveal that the E3 SUMO-protein ligase protein inhibitor of activated STAT protein y (PIASy) potentiated SUMOylation of the PXR ligand binding domain (PXR-LBD) *in vitro*. Cell-based methods were used to characterize the de-SUMOylation of PXR using expression vectors encoding six different sentrin protease (SENP) enzymes including SENP1, SENP2, SENP3, SENP5, SENP6, and SENP7. Both SENP1 and SENP2 effectively

de-conjugated both SUMO-1 and SUMO-3 from PXR, whereas SENP6 inhibited the formation of SUMO-3 chains on PXR. Collectively, the data suggest that (1) PXR can serve as a substrate for either SUMO-1 or SUMO-3 *in vitro* and in cell-based assays, (2) both SENP1 and SENP2 are able to remove SUMO moieties in a cellular environment, and (3) that SENP6 removes or prevents the formation of SUMO-chains on PXR. Taken together, the work presented in this thesis contributes to understanding the interface between PXR activators, the SUMOylation pathway and PXR activity. Future efforts should seek to determine the extent to which the biochemical mechanisms described here function in human liver and intestine in patients undergoing therapy with PXR activators. These studies are deemed critical for safe pharmacological strategies for addressing adverse drug reactions and provide a new paradigm for exploring novel approaches to repress inflammatory signaling in liver and intestine.

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List of Abbreviations

AF-1: Activation Function 1

AF-2: Activation Function 2

AR: Androgen Receptor

CAR: Constitutive Androstane Receptor

CBP: CREB Binding Protein

CCRP: Cytoplasmic CAR Retention Protein

COX2: Cyclooxygenase 2

CREB: Cyclic AMP Response Element Binding Protein

CYP: Cytochrome P450

DBD: DNA-binding Domain

DME: Drug Metabolizing Enzyme

ER: Estrogen Receptor

FOXO1/A2: Forkhead Box Transcription Factor O1/A2

FXR: Farnesoid X Receptor

G6Pase: Glucose 6 Phosphatase

GR: Glucocorticoid Receptor

GST: Glutathione S Transferase

HAT: Histone Acetyl Transferase

HDAC: Histone Deacetylase

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

HNF4α: Hepatocyte Nuclear Factor 4 Alpha

HSP90: Heat Shock Protein 90

IBD: Inflammatory Bowel Disease

LBD: Ligand-binding Domain

ICAM-1: Intercellular Adhesion Molecule 1

IFNγ: Interferon Gamma

IL: Interleukin

LBD: Ligand-binding Domain

LCA: Lithocholic Acid

LPS: Lipopolysaccharide

LXR: Liver X Receptor

MDR1: Multi-drug Resistance 1

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

NCoR: Nuclear Receptor Co-repressor

NF-κB: Nuclear Transcription Factor Kappa B

NLS: Nuclear Localization Signal

NR: Nuclear Receptor

OATP2: Organic Ion Transporting Protein 2

PCN: Pregnenolone 16α-carbonitrile

PEPCK: Phosphoenolpyruvate Carboxykinase

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

PI3K: Phosphatidylinositol 3-Kinase

PKA: Cyclic-AMP-dependent Protein Kinase

PPAR: Perioxisome Proliferator Activated Receptor

PXR: Pregnane X Receptor

PXRE: PXR Response Elements

RAR: Retinoic Acid Receptor

RIF: Rifampicin

RXR: Retinoid X Receptor

SF-1: Steroidogenic Factor 1

SMRT: Silencing-mediator for Retinoid and Thyroid Hormone Receptors

SRC: Steroid Receptor Co-activator

SREBP: Sterol Regulatory Element Binding Protein

SULT: Sulfotransferase

TNFα: Tumor Necrosis Factor Alpha

VDR: Vitamin D Receptor

XREM: Xenobiotic Responsive Enhancer Module

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Introduction

1. General Introduction to NR Superfamily

Historically, the lipophilic hormones, including steroids, retinoids and thyroids, are known as regulators of differential control of gene expression in development, cell differentiation, metamorphosis and organ physiology (1). The steroid receptors were first identified in the mid-1980s with the help of purified hormones and antibodies (2-5). The cloning of cDNAs encoding the glucocorticoid (GR) and estrogen (ER) receptors has led to the discovery of the receptors for numerous fat-soluble hormones (6). During this time, the receptors were found to exhibit high sequence similarity; hence the concept of the existence of a NR superfamily emerged. By 1990, there were a total of 15 members in the NR superfamily. To date, there are 48 functional NR family members encoded in the human genome. A group of receptors, called orphan receptors, were first found in metazoan species based on low stringency hybridization screening technique and PCR-based cloning strategies (1). However, at the time of their cloning, their physiological ligands were not known. Effort to identify the physiological ligands of orphan receptors enabled receptor "adoption" after their ligands were found. This search has led to the discovery of novel metabolic gene regulation networks and endocrine signaling pathways in animal biology (7). Members of adopted NRs include receptors for fatty acids (peroxisome proliferator-activated receptors (PPARs)), bile acids (farnesoid x receptor (FXR)), oxysterols (liver x receptors (LXRs)) and xenobiotics (PXR).

All members of NR superfamily share several conserved domains that are essential for receptor function. At the N-terminal region of NRs, the activation function 1 (AF-1) domain can act to activate transcription of target genes in a ligand-independent manner (8). It contains consensus phosphorylation sites and is responsive to several kinase signaling pathways (9). The DNA binding domain (DBD) contains two α helices and two zinc fingers that provide DNA binding specificity. The DBD of NRs binds to well-defined hormone response elements (HREs), and also imparts NR dimerization characteristics (10). Most NRs function as dimers, while some NRs are active as monomers, such as steroidogenic factor 1 (SF-1) (11). Most steroid NRs function as homodimers and bind to DNA binding sites organized as inverted repeats (12). Several important liver-enriched NRs function as heterodimers with the retinoid X receptor (RXR) including LXR, FXR, constitutive androgen receptor (CAR) and PXR. The C-terminal region of NRs includes the activation function-2 (AF-2) domain and the ligand binding domain (LBD). The LBD is connected to DBD by a flexible hinge domain which contains a nuclear localization signal (NLS). The AF-2 domain is predicted to undergo a conformational change upon ligand binding, and also serves as a binding surface for co-activator proteins necessary for transactivation (13). (Figure 1)

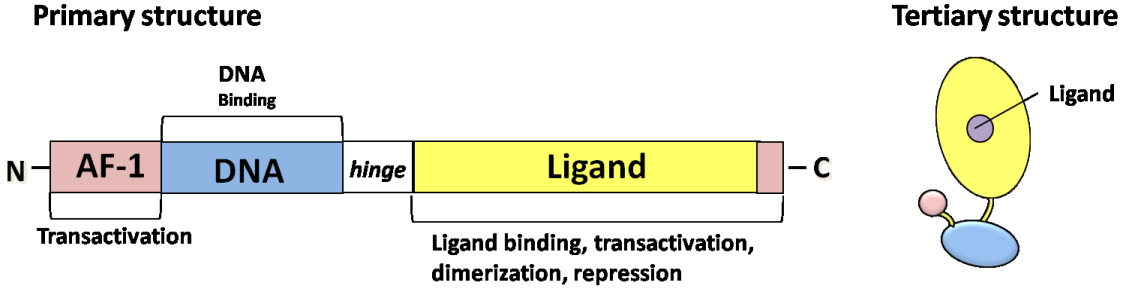


Figure 1. Domain Structures of Nuclear Receptors. Most members of the NR superfamily have a common domain structure consisting of an N-terminal AF-1, a central DBD and a C-terminal LBD.

2. NR Co-regulators

Expression cloning and functional characterization of a larger number of proteins which interact with NRs in either a ligand-dependent or independent manner have gained increasing attention (14). NR co-regulator proteins are critical components of large multi-protein complexes, including co-activators and co-repressors, which do not bind to DNA directly but have pivotal roles in regulation of target gene expression (15). Generally, genes are maintained in an off-state by recruitment of co-repressor complexes. Such complexes include nuclear receptor corepressor (NCoR) and silencing mediator for retinoic acid and thyroid hormone receptors (SMRT) (16). NCoR and SMRT do not harbor intrinsic enzymatic activity; instead they serve as the scaffold to interact with other protein co-factors which contain intrinsic histone deactylase (HDAC) activity (17). Non-liganded NRs recruit HADCs to remove acetyl groups from histone tails and induce chromatin compaction that prevents association of basal transcriptional machinery with enhancer regions of genes (18). This leads to NRs-mediated active repression of target gene expression in the non-liganded condition.

The binding of ligand to NRs results in the dissociation of the co-repressor complexes and subsequent recruitment of co-activator proteins to initiate target

gene transcription, which is called transactivation (19). Most co-activators proteins interact with NRs in a ligand-dependent manner through the C-terminal AF-2 domain via an -LXXLL- motif, where L is leucine and X is any amino acid (20). The NR-associated co-activator multi-protein complexes contain both intrinsic and extrinsic histone acetyltransferase (HAT) activity (21), a chromatin remodeling function (22), as well as mRNA elongation and splicing activity (23). Steroid receptor co-activator (SRC) proteins are co-activators for PXR transactivation. The SRC family contains intrinsic HAT activity and it also interacts with another HAT and p300/CBP-associated factor (PCAF) to form the co-activator complexes (24).

Besides ligand-induced co-regulator exchange, the activation of cell signaling cascades and phosphorylation events by protein kinase directly regulate the strength of interaction between the NRs and co-regulator multi-protein complexes (25-27). Understanding the role of cell signaling pathways that modulate NRs protein-protein interactions, DNA binding and transcriptional activity is necessary for functional implication of these signaling events.

Based on the enzymatic functions of co-regulator proteins, NRs can likely regulate gene transcription by different mechanisms, including ligand-dependent transcriptional activation, ligand-independent active repression and transrepression (28). Several important questions regarding the molecular mechanisms of NRs-mediated gene transrepression will be addressed in the next few sections of this thesis.

3. Pregnane X Receptor (PXR)

The mouse pregnane x receptor (PXR, NR1I2) was first identified in 1998 based on its sequence homology to the LBD of a number of NRs in the motif search of sequence tag databases (7). The receptor was named as PXR based on its activation by several synthetic pregnanes (7). The tissue-specific expression profiles of PXR were examined and characterized. Northern blot analysis showed that abundant level of PXR mRNA is observed in liver and at a moderate level in the intestine (29). Since the promoter which drives *Pxr* gene expression has not been well characterized, the molecular mechanisms underlying this tissue-specific expression of PXR are currently unknown. Previous studies suggest that the *Pxr* gene might be regulated by liver-enriched transcription factor hepatic nuclear factor 4 α (HNF-4α) (7,30) and/or steroid hormones (31,32).

PXR functions as a heterodimer with RXRα and is activated by numerous prescription drugs, xenobiotics, steroids and toxic bile acids (33). In fact, elucidation of the crystal structure of the LBD of human PXR revealed that it has a relatively large and spherical ligand-binding pocket which allows it to interact with a vast variety of hydrophobic chemicals (33,34). However, marked differences in the activation profiles of PXR across species exist. The rodent-specific PXR ligand pregnenalone 16α-carbonitrile (PCN) and several other naturally occurring 21-carbon steroids have little or no effect on PXR activity in human and rabbit hepatocytes. On the other hand, rifampicin (Rif), the human PXR activator, fails to induce the transcription of *Cyp3a11* which is

the prototypical PXR-target gene in mouse and rat hepatocytes (29,35). The recent availability of high-resolution PXR LBD crystal structure has provided critical clues that elucidate the basis for the observed species-specific differences in PXR activation profiles (33,36,37). The comparison of the residues in the LBD pocket of PXR across species showed that only four polar residues are different between mouse and human PXR. Recent targeted site-directed mutagenesis studies of the four key residues effectively humanized mouse PXR and conferred mouse PXR a human-like response in a cellular environment (33,38).

Upon biding with the ligand, the PXR-RXR heterodimer binds to the PXR response elements (PXREs) that are located in the CYP promoter region and activates CYP gene expression in liver (39). In addition, PXR regulates the expression of numerous other genes involved in the metabolism and transport of xenobiotic compound, including phase I oxidation enzymes carboxylesterases (CESs) (40), phase II conjugation enzymes glutathione S-transferases (GSTs) (41) and sulfotransferases (SULTs) (42), and the genes encoding phase III drug transporters organic anion transporting polypeptide 2 (Oatp2) (43) and multi-drug resistance-associated protein family (Mrp2 and Mrp3) (44) in the entero-hepatic system.

4. Physiological Functions of PXR

4.1 PXR is a Positive Regulator of Genes Encoding Enzymes that Participate in Biotransformation

4.1.1 Xenobiotic Responses

PXR is activated by a myriad of lipophilic compounds, including certain natural and synthetic steroids, bile acids, and a variety of drugs (7). It is a master-regulator of drug-inducible CYP3A gene expression in drug metabolism and detoxification and elimination of xeno-chemicals prevalent in the environment (45).

When specific xenobiotic compounds enter the liver, they activate PXR and up-regulate gene transcription for gene products responsible for xenobiotic biotransformation reactions. The catalytic action of the CYP3A family of enzymes, as well as other drug metabolizing enzymes (DMEs) in the liver, increases the water solubility of substrates mainly through oxidative metabolic reactions that result in the addition of hydrophilic groups to polar substrates. Additional PXR-target genes encode key drug transporter proteins that promote the uptake and excretion of xenobiotics to urine and bile. In this manner, PXR activation represents an adaptive response to protect the body against toxic assaults through accelerating the clearance of xenobiotics by inducing drug metabolism and drug transport (46).

PXR activation and up-regulation of CYP3A genes also form the molecular basis for an important class of drug-drug, herb-drug, and food-drug interactions in patients on combination therapy (47). Many of the xenobiotics that induce PXR are widely used prescription drugs, such as the antibiotic Rif (29), the glucocorticoid dexamethasone (32), and the antimycotic clotrimazole (48). The human CYP3A4 enzyme is involved in the oxidative metabolism of approximately 50-60% of all clinically prescribed drugs (49). Therefore, PXR-mediated activation of high levels of CYP3A in the liver would be

expected to interfere with the pharmacokinetics to increase metabolism of other co-administered drugs which are CYP3A substrates. Since many patients are likely to take more than one medication simultaneously (50), this phenomenon can be a serious problem during combination therapy, especially in patients with underlying co-morbidities that alter drug metabolic pathways. The major clinical consequence is therapeutic failure because of the undesirable lowering of the plasma level of the affected drug to non-therapeutic range (51,52).

The active compounds in natural herbal remedies contribute to inadvertent activation of PXR and thereby represent the molecular basis for a significant portion of herb-drug interactions (53-55). An example is the compound hyperforin, the active component of St John's wort (Hypericum perforatum L.), which binds to PXR with high affinity and activates CYP gene expression (56,57) to enhance the oxidative metabolism of various prescription drugs, including combination oral contraceptives, cyclosporin, and indinavir (58-63).

Food constituents also have the ability to activate PXR and are responsible for food-drug interactions (55,64). Food consisting of phytochemical mixtures, such as fruits, vegetables (65), beverages, and teas possess the ability to alter the activity of CYP3A through PXR activation (66), and modulate the metabolism of more than 50% of clinical pharmaceuticals by CYP3A (67).

Taken together, PXR activation in the liver and intestine could lead to undesirable life-threatening drug-drug and supplement-drug interactions in patients using combination therapy. Therefore, the drug development industry is employing high throughput *in vitro* and *in vivo* PXR activation and binding

assays to test novel drug candidates for their ability to induce CYP3A gene expression (68). In order to reduce adverse drug effect, the ideal drug candidate should be neutral with respect to their effects on CYP gene expression (69). The compounds might be removed from the drug development process if they are potent PXR activators (34). For example, the anti-diabetic drug troglitazone, a potent PPARγ activator (70), was withdrawn from the market due to hepatotoxicity (71). It activates PXR at the therapeutic dose (35) and its metabolites quinones catalyzed by CYP 2C8 and CYP3A4 are active intermediates in drug-induced hepatic toxicities (72-74). In the contrast, the related drugs rosiglitazone and pioglitazone, which are potent PPARγ activators and remedies for diabetes (75), do not activate PXR and form reactive quinones metabolites (76). Thus they have not shown any evidence of hepatotoxicity in patients (72,77,78).

4.1.2 PXR Regulates Bile Acid Homeostasis

In addition to a myriad of foreign chemicals, animals confront numerous endogenous toxicants whose efficient detoxification is important to animal survival (79).

Bile acids are synthesized from cholesterol in the liver and are among the first natural products isolated in pure form (80). They are present in dried extracts of bear bile, which have been used for their medicinal properties by Asian cultures for thousands of years (81,82). Bile acid synthesis provides a direct means of converting a cholesterol molecule, which is the insoluble and hydrophobic membrane constituent, to a bile acid molecule that, when ionized, is

the membrane-dissolving, water-soluble and readily-excreted detergent (83,84). The physiological roles of bile acids involve elimination of cholesterol, absorption of dietary lipids, hepatic bile formation and regulation of hepatic cholesterol synthesis (85).

The primary bile acids, including cholic acid and chenodeoxycholic acid, are synthesized in the liver through cholesterol oxidative catabolism (86). The secondary bile acids deoxycholic acid and lithocholic acid (LCA) are formed in the intestine from primary bile acids (87). The synthesis of a full complement of bile acids is a complex process, requiring 17 enzymes (84,88) and the multi-step conversion of cholesterol to bile acids confers the detergent-like properties to the bile acids that are essential for their physiological functions (89).

The expression of selected enzymes in the bile acids synthesis pathway is tightly regulated by NRs and other transcription factors, which ensure a constant supply of bile acids and the maintenance of hepatic cholesterol catabolism (83). While FXR (NR1H4) and LXR (NR1H3) play a fundamental role in regulating bile acid and cholesterol levels in the liver (79,90), PXR is also involved in the regulation of biosynthesis, transport and metabolism of bile acids that are extremely toxic at excessive concentrations occurring during hypercholesterolemia and cholestasis (79,91).

The rate limiting step in bile acid formation is the 7-hydroxylation of cholesterol by CYP7A1 enzyme (92). PCN treatment negatively regulates *Cyp7a1* expression in mice, which suggests that PXR activation blocks bile acids synthesis (93). Among the genes that are up-regulated by PCN treatment are

Oatp2 and *CYP3a11* (43,45). The CYP3A11 enzyme catalyzes the hydroxylation of bile acids, and OatP2 transports bile acids across the sinusoidal and canalicular membranes of hepatocytes (84) (Figure 2).

Since PXR regulates key enzymes in bile acid PXR biosynthesis, transport and metabolism, it provides a fundamental molecular basis for Rif treatment in patients suffering from chronic cholestatic liver disease (43,94). Cholestasis, a pathogenic state characterized by cessation or impairment of bile flow and the accumulation of toxins normally excreted in bile, can cause nutritional imbalance and irreversible liver damage (95). LCA is a highly toxic secondary bile acid that causes cholestasis when accumulated in the body (43,79). The harmful effects of LCA and other bile acids are attenuated by two mechanisms, namely hydroxylation and conjugation, for elimination and detoxification of bile acids (96). LCA at the pathophysiological level binds to PXR, whose activation down-regulates *Cyp7a1* to block bile acids synthesis, accelerates metabolism catalyzed by CYP3A and promotes uptake of bile acids from the blood by induction of *OatP2* (43,97). These reactions make LCA more hydrophilic and facilitate the excretion of bile acids in the urine or feces. Therefore, PXR is critical as an endogenous bile acid receptor and regulates bile acid homeostasis in bile acid elimination and detoxification. Potent PXR ligands may be efficacious in the treatment of cholestasis and other hepatic diseases (98).

Figure 2

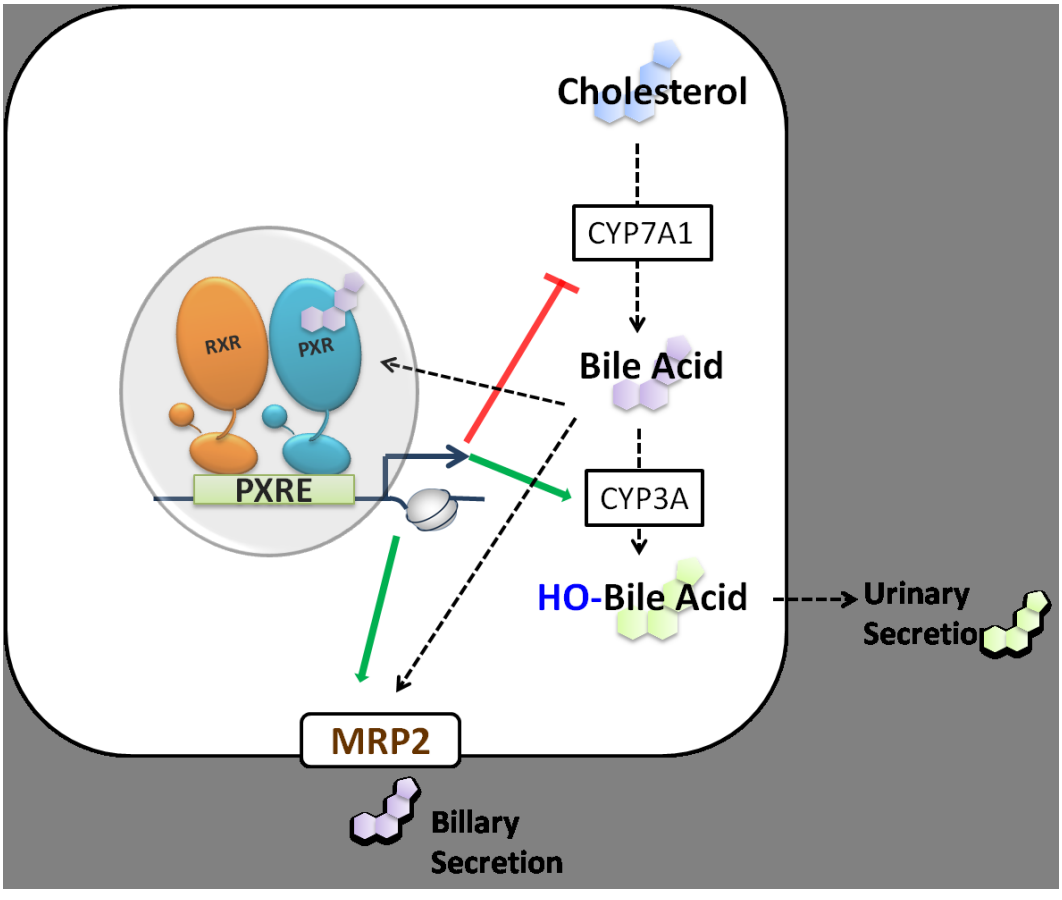


Figure 2. Regulation of Bile Acid Homeostasis by PXR. PXR is activated by bile acids and regulates genes involved in the biosynthesis, transport and metabolism of cholesterol and bile acids. Genes up-regulated by PXR are indicated by the green arrows. Genes down-regulated by PXR are indicated by the red line. PXRE, PXR response element; MRP2, Multidrug resistance associated protein 2.

4.2 Negative Physiological Functions of PXR

Besides the involvement in the regulation of xenobiotics/endobiotics metabolism and transport, PXR also governs ligand-dependent repressive effects upon gluconeogenesis, lipid β-oxidation and subsequent ketogenesis, and the inflammatory response, likely through crosstalk with other signaling pathways controlling these essential biological functions.

4.2.1 Glucose Homeostasis

Hepatic glucose production is tightly regulated by insulin and glucagon signaling pathways which play a major role in animal survival during fasting and starvation. Genes that are involved in gluconeogenesis include *glucose-6-phosphatase (G6Pase)* and *phosphoenolpyruvate carboxykinase (Pepck)*, which are the rate-limiting enzymes that control the serum level of glucose (99). In the liver, gluconeogenesis is positively regulated by glucagon, cAMP and glucocorticoid, which increase glucose production by induction the transcription of *G6Pase* and *Pepck*, whereas insulin and glucose suppress the expression of these genes (100).

Ligand-mediated activation of PXR interferes with the regulatory gene networks that control glucose homeostasis. For example, PCN decreases blood glucose levels in fasting wild-type mice but not in PXR-null mice (101). *Pepck1* and *G6Pase* genes are reduced in the transgenic mice expressing constitutively activated PXR in the liver and intestine (102). These observations are in consistent with the result that PXR activation down-regulates the transcriptional activity of FoxO1, a positive regulator of gluconeogenic genes (103).

FoxO1 is a member of the fork head family of transcription factors which are characterized by the fork head domain (104). The de-phosphorylated form of FoxO1 positively regulates the transcription of genes involved in gluconeogenesis. Insulin exerts a repressive action on FoxO1 by activating the phosphatidylinostiol-3-kinase (PI3K)-Akt pathway to increase phosphorylation of FoxO1 (105). The phosphorylated FoxO1 protein is then sequestered in the cytoplasm to remain transcriptionally inactive (106). Additional studies reveal crosstalk between FoxO1 and PXR (103,107). Ligand-dependent activation of PXR prevents FoxO1 binding to its insulin response sequences in target genes such as *G6Pase* and *Pepck*, thereby inactivating FoxO1 transcriptional activity (100,103).

Glucose production is also mediated by liver-enriched hepatic nuclear factor 4α (HNF4α) transactivation through its recruitment of co-activator protein, PPARγ coactivator-1α (PGC-1α) (108). Ligand-activated PXR interferes with the interaction of HNF4α with PGC-1α to suppress HNF4α transactivation (109). This represents another mechanism by which PXR ligand-dependent activation might suppress hepatic gluconeogenesis.

Finally, it has been proposed that PXR could interfere with the cAMP-response element binding protein (CREB) signaling pathway (101). CREB is phosphorylated by cAMP-dependent protein kinase (PKA) in response to glucagon secretion during fasting state. CREB binds to cAMP-responsive elements (CRE) to activate the transcription of genes encoding gluconeogenic enzymes G6Pase and PEPCK1 (110). PXR binds to CREB to prevent the

interaction between CREB and CRE on the promoter regions of these genes, thus repressing CREB-mediated gene activation programs.

In this manner, the crosstalk between the signaling pathways that control glucose homeostasis and drug metabolism demonstrate that PXR directly interacts with other transcription factors and accessory proteins, such as FOXO1, CREB, and PGC-1α, all of which are critical for efficient gluconeogenic gene transcriptional regulatory circuits (111).

4.2.2 Lipid Metabolism

As described above, liver is the major site for drug metabolism. Liver is also the organ that metabolizes lipids to produce energy in the form of ATP from fat during the fasting and starvation response. Hepatic lipid homeostasis involves balanced lipid synthesis (lipogenesis), fatty acid catabolism (β-oxidation), lipid uptake and secretion (102). When blood glucose is low, liver provides the energy sources in the form of ketone bodies (acetoacetate and 3-hydroxybutylate) to the extra-hepatic tissues via β-oxidation of fat and ketogenesis from the Krebs cycle (112). Recent clinical observations reveal that PXR activation affects lipid metabolism in patients treated with PXR activators. For example, treatment with the antibiotic and PXR activator Rif induces hepatic steatosis in tuberculosis patients (113). Transgenic mice expressing constitutively active PXR exhibit marked triglyceride accumulation (114).

The role of PXR in the development of hepatic steatosis is linked to expression of transcription factors and enzymes that function in lipogenesis, such as stearoyl-CoA desaturase 1 (SCD1), a key enzyme in the synthesis of

unsaturated fatty acids (115). Insulin increases the transcription of *SCD1* by activating the lipogenic transcription factor sterol regulatory element-binding protein (SREBP) (116). But, PXR activation-induced hepatic lipid accumulation in transgenic mice is independent of SREBP, and is linked to increased expression of the free fatty acid transporter *Cd36* and accessory lipogenic enzymes such as SCD1 and long-chain free fatty acid elongase (102,117). *Cd36* has been shown to be regulated by oxidized low density lipoprotein and long chain fatty acids (118). *Cd36* is a previously indentified PPARγ target gene (119), and this study showed that PXR may increase the expression of *Cd36* by directly binding to the *Cd36* promoter. In fact, there is a direct repeat spaced by three nucleotides spacer (DR-3) PXRE in the mouse *Cd36* promoter, which suggests that *Cd36* serves as a direct transcription target of PXR and it can be activated by both PXR and PPARγ to regulate lipid homeostasis (102).

PXR activation also provokes suppression of several genes whose gene products function in β-oxidation. Treatment of mice with PCN inhibits lipid oxidation by down-regulating the expression of carnitine palmitoyltransferase 1α (CPT1α) and mitochondrial 3-hydroxy-3-methylglutarate-CoA synthase 2 (HMGCS2), two key enzymes involved in β-oxidation and ketogenesis. Importantly, PXR-mediated down-regulation of these two genes is present in wide type but not PXR-null mice. It has been shown that activated PXR can physically interact with Forkhead box A2 (FoxA2) and prevent FoxA2 binding to the *Cpt1a* and *Hmgcs2* gene promoters, thereby rendering FoxA2 transcriptionally inactive.

In addition, ligand-activated PXR interferes with HNF4α by targeting the PGC-1α co-activator protein to function in lipid homeostasis. HNF4α and PGC-1α jointly regulate the expression of CPT1α (120). Besides the interaction with FoxA2, PXR interferes with HNF4α in the regulation of CPT1α as well. Therefore, PGC-1α represents a coordinate point of crosstalk between PXR and other signaling pathways (100).

4.2.3 Inflammatory Response

Exposure to xenobiotic chemicals can impair immune function. Treatment of patients with Rif suppresses humoral and cellular immune responses in liver cells (121). Rif's immunosuppressive role has been well described in humans since 1970s (122-125). It had been suggested that the immunosuppressive effects were mediated by Rif acting as a ligand and activator for the GR (126). But the result was not supported by other groups who showed that Rif is not a biologically significant activator for GR (126,127).

After PXR (NR1I2) was characterized and cloned in 1998 (29), Rif is identified as a potent ligand and activator of human PXR (7). Considering that human PXR activates CYP3A, these findings help to explain the prior reported studies that CYP3A is the major CYP gene induced by Rif in human primary hepatocytes (128). But the mechanism through which Rif exerts immunosuppressive effects remains unknown. Hepatic P450 activity is down-regulated by various infectious and inflammatory stimuli, primarily due to inhibition of P450 gene transcription (129). Since PXR is a mater-regulator of CYP3A gene, it has been hypothesized that inflammation could suppress PXR

transcriptional activity. Recent reports have described mutually repressive and negative crosstalk between the PXR and NF-κB signaling pathway, thus providing a potential molecular mechanism that links xenobiotic response and inflammation (130-132) (Figure 3).

There are five members in the NF-κB family, namely p65 or Rel A, Rel B, c-Rel, p50, and p52 (133). NF-κB is a key regulator of inflammation and the innate and adaptive immune responses. Under basal condition, p65 is normally sequestered in the cytoplasm by the inhibitory protein inhibitor of NF-κB (IκB). (133). In response to activation signals, such as inflammatory stimuli, pro-inflammatory cytokines, reactive oxygen species and viral products, downstream signaling events induce phosphorylation, ubiquitination, and proteosome-dependent degradation of IκB and release NF-κB to translocate to the nucleus where it regulates the expression of inflammatory genes. (134)

Activation of PXR by Rif suppresses the expression of typical NF-κB target genes, such as cyclooxygenase 2 (COX-2), tumor necrosis factor alpha (TNFα), intercellular adhesion molecule 1 (ICAM-1 or CD54), and several interleukins. (130) Conversely, NF-κB target gene expression is elevated in hepatocytes derived from the PXR-null mice compared to that from the wild type animals (132). The PXR-null mice also exhibit a prominent and increased small bowel inflammatory infiltrate. (130) These observations indicate that PXR is involved in suppressing the NF-κB-regulated gene expression and deregulation of PXR expression or activity may make the gastrointestinal tract susceptible to inflammatory injuries (100).

In turn, inflammatory stimuli decrease xenobiotic metabolism capacity in human and experimental animals. One example is the bacterial toxin lipopolysaccharide (LPS), which activates NF-κB through a sequential TLR4 intracellular signaling cascade. (135). Another example is the inflammatory cytokine TNFα. It binds to its receptor (TNFR) and recruits TNFR1-associated death domain protein (TRADD), TNF receptor-associated factor protein (TRAF2) and other signaling transducer proteins, which lead to translocation of NF-κB into the nucleus (136). LPS and TNFα-induced NF-κB activation have been shown to suppress *CYP3a4* gene expression. NF-κB p65 subunit directly interacts with the DBD of RXRα and inhibits its binding to the consensus DNA sequences. Because PXR functions as a heterodimer with RXRα, the transactivation by the PXR-RXR complex is down-regulated in response to NF-κB signaling pathway (131).

Figure 3

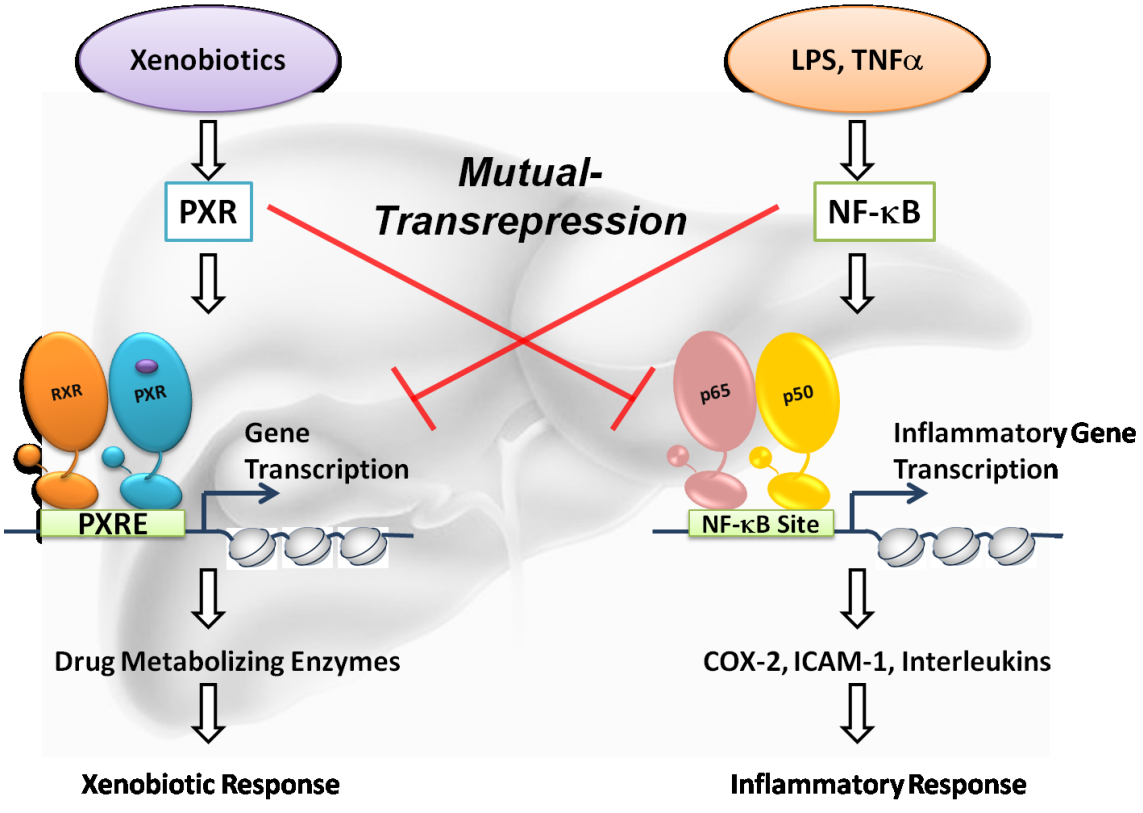


Figure 3. Mutual transrepression between PXR and NF-κB signaling pathway. The PXR and NF-κB mutual suppression may link xenobiotic response and inflammation in the hepato-intestinal axis. COX-2, Cyclooxygenase 2; ICAM, intercellular adhesion molecule; LPS, lipopolysaccharide; NF-κB, nuclear factor kappa B; PXR, pregnane X receptor; PXRE, PXR response element; TNF, tumor necrosis factor.

5. Mechanisms of PXR Transcription Regulation

Considering the distinct regulatory roles of PXR in several physiological conditions, we next discuss its different mechanisms of transcription regulation, including both transactivation and transrepression.

The most prevalent activation of CYP3A by PXR is mediated by a linear series of events. It was previously believed that human PXR resided exclusively to the nucleus even in the absence of ligand (137). However, other studies have detected cytoplasmic localization of PXR and ligand-dependent translocation of PXR from the cytoplasm to the nucleus after binding with a ligand (138,139). Further evidences demonstrate the interaction of PXR with heat shock protein 90 (HSP90) and cytoplasmic CAR retention protein (CCRP) in the cytoplasm of liver cells (139). Upon ligand binding, PXR dissociates from the CCRP-HSP90 complex and translocates to the nucleus where it binds as a heterodimer with RXRα to the DNA response element composed of two copies of the consensus NR binding motif AG (G/T) TCA (34,140). The response elements are organized as direct repeats with 3 to 5 nucleotides separating the DBD binding sites (DR-3, DR-4, and DR-5 elements), as well as everted repeats separated by 6 or 8 nucleotides (ER-6 and ER-8 elements) (29).

The full functional activity of PXR is linked to the recruitment of co-regulator proteins that have pronounced effect on gene expression through protein-protein interaction instead of binding to DNA directly. Generally, non-liganded PXR interacts with co-repressor complexes including NCoR/ SMRT and HDACs (141,142). The co-repressor complexes exert inhibitory

effect on transcriptional activity and maintain chromatin structure in the compact state, thus reducing the accessibility of enhancer region of target gene to the necessary basal transcriptional machinery. For example, un-liganded PXR interacts with SMRT to repress the basal transcription of CYP3A gene which is reversed by PXR ligand paclitaxel (143). In the presence of ligand, the C-terminal AF-2 domain undergoes a conformational change that favors binding with co-activator proteins via the –LXXLL- motif. The co-repressor protein complexes are dissociated and multi-protein co-activator complexes including HAT are recruited subsequently (Figure 4). In this process, TBL1/TBLR1, the component of NCoR co-repressor complexes (144), serves as the NR co-repressor/co-activator exchange factor (N-CoEx) (145). It functions as the adaptor factor to recruit the ubiquitin conjugating/19S proteasome complex that targets the co-repressor complexes for degradation after ligand binding (145).

In the contrast to transcriptional activation, which usually involves the binding of PXR to its HRE in the promoter region of CYP3A gene, the inhibition of NF-κB activity does not require PXR to directly bind to typical response elements. Transrepression is widely involved in negative regulation of gene expression. Previous results suggest that PXR may negatively regulate inflammatory response through protein-protein interaction in a SUMOylation-dependent pathway. But this SUMOylation-dependent transrepression mechanism remains to be further identified.

Figure 4

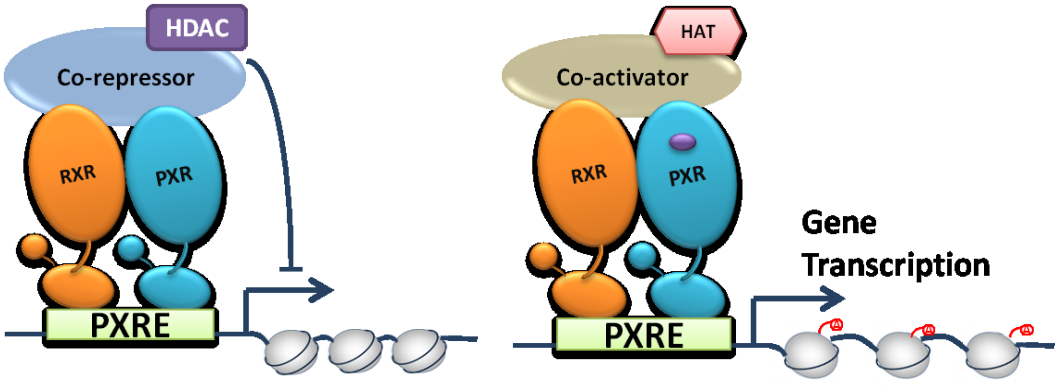


Figure 4. PXR activity is regulated by different co-factor interactions. PXR interacts with co-repressor complexes which recruits HDACs. This inhibits transcriptional activity through promoting chromatin compaction and reducing accessibility of genes to basal transcriptional machinery. Ligand binding induces a conformational change and dissociates the co-repressor complexes. The co-activator complexes with HAT activity are recruited to promote histone acetylation and enhance gene transcription.

There are several models of NRs-mediated transrepression: first, NRs regulate key components in the NF-κB and AP-1 signal-transduction pathways (146-148); second, NRs compete with NF-κB and AP1 for limiting amount of co-activators (149,150); third, NRs alter NF-κB and AP1 co-regulator complex composition (151,152); and last, NRs modify basal transcription machinery (153,154).

Several recent reports indicate a general molecular strategy for SUMOylation of a subset of NRs that transrepress immunity in a signal and gene-dependent manner. For example, a SUMOylation-dependent pathway has been identified in which PPARγ mediates the transrepression of inflammatory

response genes in mouse macrophages. Ligand induced SUMO modification of PPAR-γ and then the SUMOylated ligand-bound PPAR-γ inhibits gene transcription by preventing signal-dependent recruitment of ubiquitin-19S proteasome complex required for NCoR clearance from inflammatory gene promoter/enhancer region. Subsequent research in LXRs indicates that SUMOylation is required for the repression of interferon-γ (IFN-γ) response genes in brain astrocytes. SUMOylated LXRs form complexes with SUMO E3 ligases and signal transducer and activator of transcription 1 (STAT1), thus preventing STAT1 binding to DNA in response to IFN-γ stimulation.

Importantly, previous results from our lab have demonstrated that PXR is SUMOylated in response to inflammatory stimulation and represses the NF-κB target gene expression (132). These findings raise our interest in further studying PXR SUMOylation/de-SUMOylation mechanisms to unravel the importance of this post-translational modification in inflammatory response.

6. PXR SUMOylation

The SUMOylation pathway results in the covalent attachment of SUMO to substrate proteins. SUMO is a small polypeptide, ~10kD in size. The first SUMO protein found was the *Saccharomyces cerevisiae* suppressor of mif two 3 homolog 1 (SMT3) (155). Based on two-hybrid interaction screening and cloning, SUMO proteins have been found to be present in plant, yeast, fly and other species. Within mouse and human, there are four types of SUMO proteins, named as SUMO-1, -2, -3 and -4. However the functionality of SUMO-4 remains currently unclear, so that SUMO-4 will not be discussed in this thesis. SUMO-1,

-2 and -3 are all highly conserved during the evolutionary process (156). SUMO-2 and SUMO-3 share 97% sequence identity so that they are always termed as SUMO-2/3, while SUMO-1 shares only 50% sequence identity with SUMO2/3 (Figure 5A).

The SUMO proteins have C-terminal extension of 2-11 amino acids. They are expressed in the immature forms initially and need C-terminal extension removed by SUMO-specific proteases (SENPs) to expose the invariant glycine-glycine motif for maturation. After the maturation process, the mature forms could enter the three-step SUMOylation pathway. The first step is the activation of the mature SUMO protein by the SUMO-specific heterodimeric E1 activating enzyme, AOS1-UBA2 complex, which has both adenylation and thioesterifiation functions (157,158). The ATP-dependent reaction forms a thioester bond between the C-terminal carboxyl group of glycine and a catalytic cysteine residue in E1 (159). The second step involves transferring the SUMO protein from the E1 activating enzyme to the E2 conjugating enzyme, Ubc9, which leads to a new thioester bond between the C-terminal carboxyl group of SUMO and a catalytic cysteine residue in E2 (160). Efficient modification by SUMO protein, especially *in vivo*, further requires the action of specific SUMO E3 ligases, which catalyze the shift of SUMO to the target and the formation of an isopeptide bond between the glycine residue of SUMO and the lysine residue of the target (161) (Figure 5B).

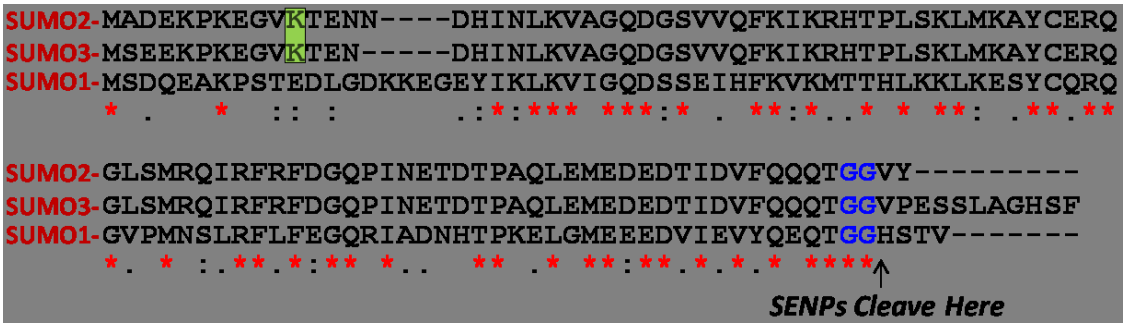
Several E3 ligases have been identified. The first E3 ligase found in yeast was Siz1(162) and the mammalian PIAS proteins have been found to increase the rate of SUMO conjugation *in vivo* subsequently (163). There are at least four

subtypes of PIAS proteins, namely 1, 3, x and y (164). The PIAS family has a central RING-FINGER-like motif that binds to targets and Ubc9 directly and interacts with SUMO through a non-covalent SUMO-interaction/binding motif (SIM) (165). The nucleoporin Ran-binding protein 2 (RanBP2) represents another type of E3 ligase (166). It increases the rate by positioning the thioester bond between SUMO and Ubc9 to an optimal orientation for attack by substrate (167) instead of directly interacting with substrate. In addition, the human Polycomb group member Pc2 (168) and histone deacetylase HDAC4 (169) have also been recently found to function as E3 ligases.

The acceptor sites on SUMO targets follow a consensus sequence. It is shown to be Ψ-K-x-D/E, where Ψ is a hydrophobic amino acid, K is the lysine residue, x is any amino acid, and D/E is an acidic residue. Many proteins carry the consensus SUMOylation sequence, even the SUMO proteins themselves. The N-terminal extension of SUMO-2/3 has the SUMO acceptor site and is targeted by E1, E2 and E3 to form polymeric chains. This feature is not shared by SUMO1, since it does not contain the consensus SUMOylation sequence on itself. The distinct chain formation capacity may suggest in part different roles and functional consequences of modification by SUMO-2/3 versus SUMO-1 which will be discussed in the following sections.

Figure 5

A)



B)

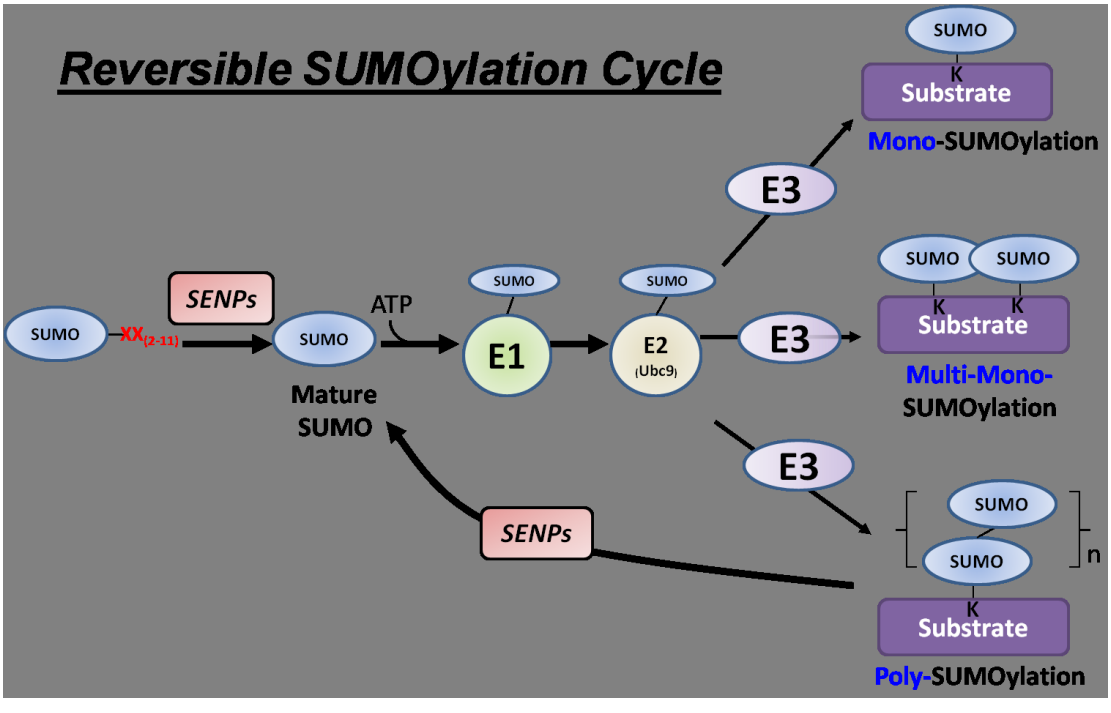


Figure 5. PXR Reversible SUMOylation Pathway. A) The sequences of human SUMO-1, -2, and -3 are compared. Similarities are indicated by red asterisk. The consensus SUMOylation site (K11) on SUMO-2/3 is shown with green box. Sentrin proteases cut C-terminal extensions to expose the glycine-glycine motif. **B)** The mature SUMO protein is incorporated into

its specific substrate by a cascade of enzymes termed as E1, E2, and E3 to produce SUMO-modified substrates in a signal-dependent manner. Specific sentrin protease enzymes then cleave the SUMO moiety from substrates and allow the SUMO moiety to recycle.

The SUMOylation pathway is highly dynamic and reversible. De-SUMOylation is potentially catalyzed by SENPs, which belong to the family of cysteine proteases with conserved C-terminal catalytic domains. Mammalian genomes encode up to seven SENPs, designated SENP1-3 and SENP 5-8 (Figure 6A). Although SENP8 shares the conserved catalytic domain, it has been shown to be a NEDD8-specific protease and lack the ability to process or de-conjugate SUMO substrates (170). Other six SENPs contain both endopeptidase and isopeptidase activity for de-conjugating SUMO from substrates (Figure 6B).

Based on their functional activities and preferences towards targets, mammalian SENPs can be classified into three groups. SENP1 and SENP2 are able to target all SUMO isoforms and function both in their processing and de-conjugation pathways. SENP3 and SENP5 regulate the modification of monomeric SUMO-2/3 and to a less extent SUMO-1 conjugates. SENP6 and SENP7 also regulate preferentially on SUMO-2/3. Neither SENP6 nor SENP7 act on maturation of SUMO protein precursors and they show no activity in the de-conjugation of monomeric SUMO-2/3 from substrates. Rather, the main role of SENP6 and SENP7 is to de-conjugate poly-SUMO-2/3 chains. SENPs can also be distinguished based on their different sub-cellular localizations. SENP1 resides in the nucleus, excluding the nucleolus (171), whereas SENP2 localizes to the nuclear envelope (172). SENP3 and SENP5 localize to the nucleolus

(173,174), whereas SENP6 localizes to the cytosol (175). And SENP7 localizes to the nucleoplasm (176).

Figure6

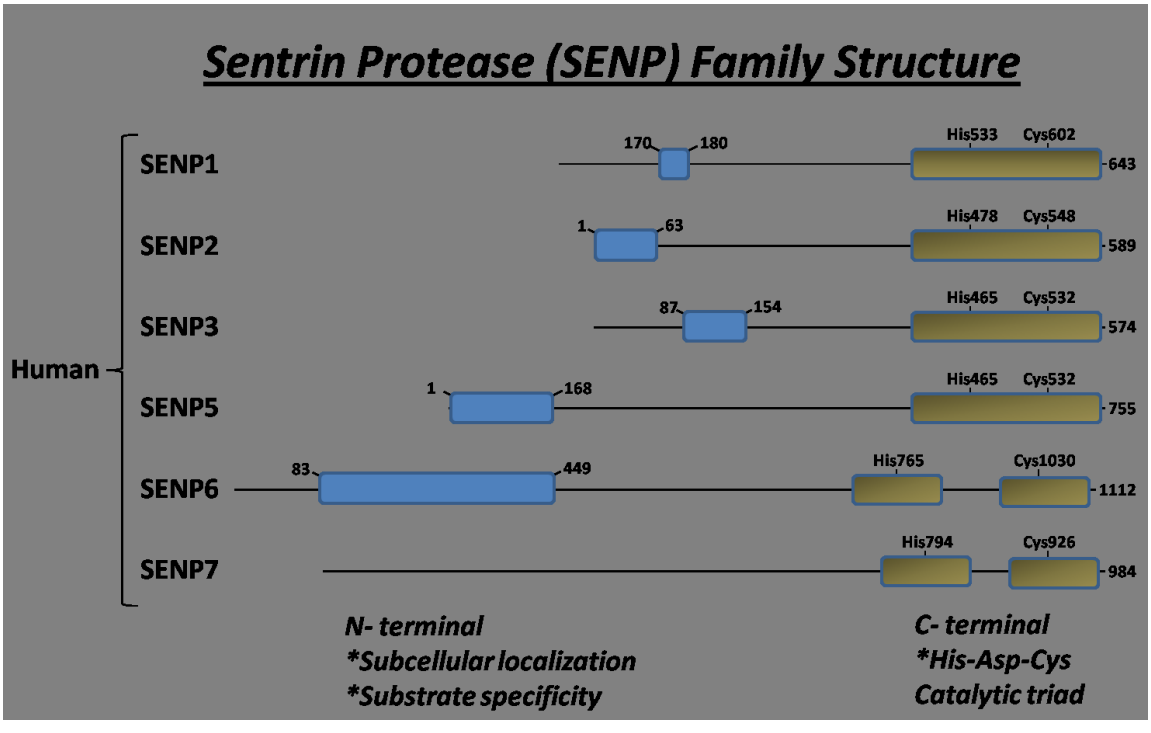


Figure 6A). The Sentrin Protease Family. Each SENP family member contains a C-terminal catalytic domain. Both SENP6 and SENP7 contain additional sequences within their C-terminal domain. The N-terminus of each SENP family member contains sequences that impart both sub-cellular localization and substrate specificity.

B)

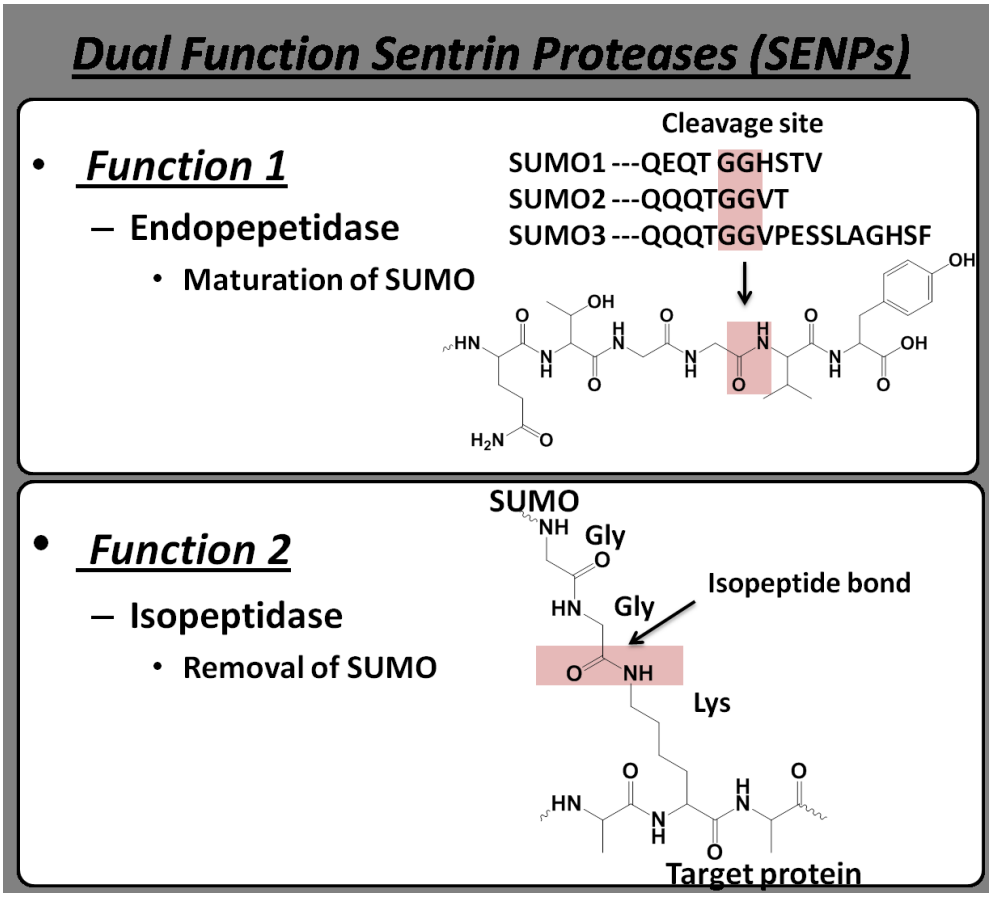


Figure 6B). The SUMO-specific Proteases Dual Functions. Certain SUMO-specific proteases (SENPs) are responsible for SUMO precursor maturation and cleavage at the C-terminal extension of SUMO. They also cleave at the isopeptide bond between the terminal glycine of mature SUMO and the lysine of a substrate. The sites of cleavage by SUMO proteases are highlighted in pink and the scissile bonds are indicated by the arrow.

Previously, the PXR protein has been shown to be modified by SUMO-2/3 in response to TNFα signaling in hepatocytes. The SUMOylated PXR protein incorporated SUMO-2/3 chains and the feedback repressed the immune response in hepatocytes (132). Therefore, SUMOylation of PXR represents the likely molecular basis underlying the inhibition of hepatic immune response in Rif-treated patients. Here, we further study the mechanism of the PXR SUMOylation and the transrepression mechanism of inflammation. By integrating our previous data, the working model has been proposed (Figure 7). PXR is SUMOylated by SUMO-1 and/or SUMO-2/3 in response to an inflammatory stimulus in a ligand-dependent and/or ligand-independent manner. The SUMOylated PXR prevents co-repressor complex dissociation from NF-κB through protein-protein interactions. In order to test the hypothesis, NF-κB target gene expression was analyzed in Hepa1-6 cell line, including COX-2, IL-6 and IL-1rn. We also used cell-based methods to compare SUMO-1 modification capacity with SUMO-3 and an *in vitro* SUMOylation assay to determine the PXR SUMOylation reaction mechanisms. Furthermore, we described the substrate preferences of six SENPs and indentified possible SUMOylation site(s) based on site-directed mutagenesis. Taken together, the work presented in this thesis contributes to understanding the interface between PXR-mediated gene activation, the SUMOylation pathway and inflammation.

Figure 7

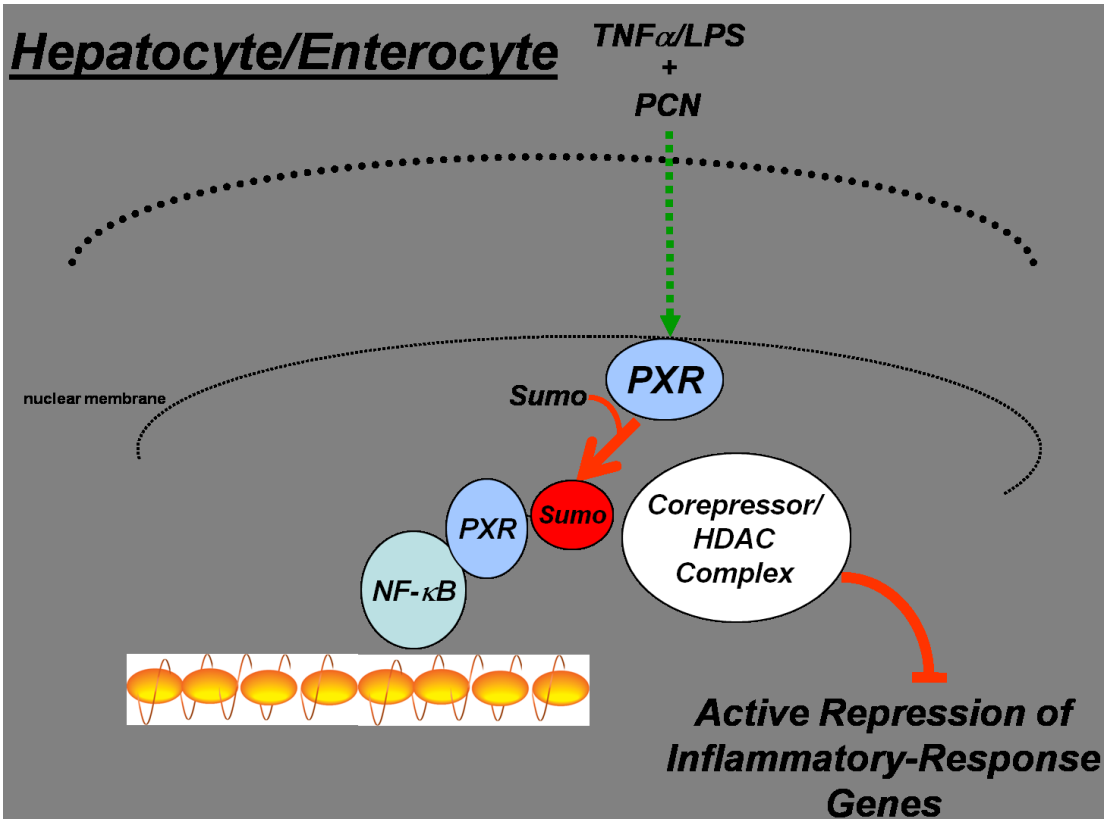


Figure 7. Model of PXR-mediated Transrepression of Inflammatory Response Pathway. Co-stimulation of cells with PXR activators and inflammatory stimuli leads to the formation of trans-repressive multi-protein complexes on the promoters of specific inflammatory-response genes.

Materials and Methods

Cell Culture and Treatment of Hepa1-6 Cells.

The Hepa1-6 cell line is a readily available immortalized hepatocyte cell line isolated from mouse hepatocytes by Darlington et al., in 1980 (177). Studies in our laboratory have confirmed that induction of Cyp3a11 and expression of PXR is absent in this cell line (data not shown). The Hepa1-6 cell line has been used successfully in a variety of studies such as cell hybridization, biochemical analysis of tissue-specific gene products, and the modulation of expression of genes governing differentiated phenotypes. Hepa1-6 cells were maintained in DMEM supplemented with 10% FBS supplemented with pen-strep. Sub-culturing was performed at ~70% confluence using a 1:5 dilution. Cells were transfected with the indicated plasmids using lipofectamine 2000 per manufacturer's instructions (Invitrogen). Cells were were with treated with 10 micromolar pregnenalone 16a-carbonitrile (PCN), a well-known mouse PXR agonist, for 24 hours. Treatment of cells was continued with lipopolysaccharide (LPS, 10 micrograms/ml media) or tumor necrosis factor alpha (TNFa, 10 nanograms/ml media) in the presence and absence of PCN for an additional 12 hours.

RNA Isolation and q-PCR Analysis

Total RNA was isolated from cell culture using a commercially available kit (Rneasy, Qiagen) according to the manufacturer's instructions. RNA was visualized by electrophoresis to ensure its integrity. Isolated RNA was DNase

treated (Sigma), reverse transcribed (Promega), and quantitative PCR was performed using an Applied Biosystems StepOne Plus real-time PCR system to detect mRNA expression specific for indicated genes. Fold induction was calculated using beta-actin mRNA as a normalization control.

Bacterial Expression and Purification of GST-tagged Fusion Proteins

GST fusion protein expression vectors were transformed into BL21DE3 cells (Novagen #69450-1), which contain the T7 polymerase stably intergrated under the control of an IPTG inducible promoter. After plated the plasmid on LB-amp (100ug/mL) plates, a single colony was used to inoculate a 10 mL LB liquid culture containing 100ug/mL ampicillin. This culture was grown at 37°C shaking for 6 hours. A 15% glycerol stock was prepared from the 10mL culture in a final volume of 0.5 mL. Transformants were inoculated and grown as before for 10 hours. A 50 mL culture was then inoculated and grown as before for 2-3 hours. The culture was induced to express the fusion protein with 0.5 mM IPTG (0.24g/L) for 4-6 hours. The cells were pelleted and resuspended in 5.0mls NETN (NETN=100mM NaCl, 20 mM tris pH=8.0, 1 mM EDTA, 0.5% NP-40). The cells were sonicated 3 times, on a medium setting, for 1 minute each on ice. The sonicated cell lysates were centrifuged at 12,000 rpm for ten minutes at 4°C. The supernatant was made 10% in glycerol and frozen at -80°C for future use. 25 µl of glutathione agarose beads (Pharmacia #17-0756-01) was added to the 300 µl supernatant of the -80°C frozen supernatant after thawing and incubated with shaking at 4°C for 30minutes in a 1.5 mL eppendorf tube. The beads were pelleted for 1 minute and then

washed three times in a 1mL NETN.

***In Vitro* SUMOylation Assay**

Each SUMOylation reaction (Enzo Life Sciences Inc) contained 1 μM recombinant purified PXR in total 20-μl volume in the presence or absence of Mg2+-ATP. The assay components including SUMO proteins, E1, E2 and E3 enzymes were mixed in a microcentrifuge tube and incubated at 30°C for 60 min, and the reaction was quenched by the addition of 20 μl of 2× SDS-PAGE gel loading buffer. To detect the SUMOylated proteins, a 5-μl sample of each reaction was resolved by using 10% SDS-PAGE, and the immunoblot analysis was conducted by using anti-PXR antibodies (H-11, Santa Cruz).

Cell-Based SUMOylation Assay

Plasmids pcDNA3-6X-His-SUMO1, and pcDNA3-6His-SUMO3 were kind gifts from Dr. Ronald T. Hay (University of Dundee, Dundee, United Kingdom). For transfection assays, Hepa1-6 cells were grown in six-well dishes for 24 h until 80% confluence. Cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). Forty-eight hours after transfection, cells were washed twice with phosphate-buffered saline and harvested in 200 μl of lysis buffer (6 M guanidinium-HCl, 10 mM Tris, 100 mM sodium phosphate buffer, pH 8.0). After sonication the cell lysates were cleared by centrifugation at 3,000 x g for 15 min. The cleared cell lysates were mixed with 25 μl of Cobalt-linked agarose (QIAGEN) that had been prewashed three times in cell lysis buffer. The mixture was incubated for 2 h on a rotator at room temperature and centrifuged for 2 min at 1,000 rpm to gather the beads. The beads were washed once in wash buffer I (8 M urea, 10 mM Tris, and 100

mM sodium phosphate buffer, pH 8.0), three times in wash buffer II (8 M urea, 10 mM Tris, 100 mM sodium phosphate buffer, pH 6.3, 0.1% Triton X-100, and 5 mM β-mercaptoethanol, and once in wash buffer III (150 mM NaCl, 10 mM imidazole, and 50 mM sodium phosphate buffer, pH 6.75). The beads were resuspended in 40 μl of 2× SDS-PAGE gel loading buffer and boiled for 5 min, and 20-μl samples were resolved by using 10% SDS-PAGE. The gel was transferred to polyvinylidene difluoride membrane using standard methods, and immunoblot analysis was performed to detect the SUMOylated form of PXR using the H-11 monoclonal anti-PXR antibody.

Results

1. Expression of PXR Transrepresses the Induction of Endogenous Inflammatory Response Genes in Hepa1-6 Cells.

The NF-κB transcription factor is a critical regulator of the host immune and inflammatory response in various disease states. We analyzed the NF-κB target gene mRNA levels by RT-qPCR in Hepa1-6 cell line, which is a derivative of the BW7756 mouse hepatoma arose in a C57/L mouse. As there is no endogenous PXR protein expressed in Hepa1-6 cell line, we transfected the cells with mouse PXR or mock plasmid as indicated and treated with mouse PXR activator PCN for 24 hours. Cells were then treated for an additional 12 hours with LPS in the presence and absence of PCN as indicated. We isolated total RNA and examined the relative expression levels of several known NF-κB target genes (Figure 8).

Previous study of the expression levels of genes encoding TNFα, IL-6, IL-1α, and IL-1β showed that these genes are significantly induced when PXR is ablated in PXR-KO mice (132). To confirm the involvement of PXR in anti-inflammation process, we first tested inflammatory response genes in Hepa1-6 cells in the absence of PXR.

Among the four non-transefected groups (Veh, PCN, LPS, PCN+LPS), analysis of the expression levels of genes encoding Cox-2, IL-6 and IL-1rn using RT-qPCR showed that their levels were significantly increased in LPS-treated group. These results reveal an active role for 12h LPS treatment in inducing the expression of genes that encode key inflammatory cytokines. Also, in the

absence of mouse PXR, there was no difference between LPS and PCN+LPS co-treatment groups, which proved that Hepa1-6 cell line is an ideal cell model to study PXR-dependent effect.

In order to investigate the repressive role of PXR on NF-κB-mediated inflammatory signaling pathway, we analyzed the gene expression levels in mouse PXR-transfected groups. When mouse PXR was activated by PCN, the endogenous inflammatory cytokines were repressed about one fold. When cells were challenged with LPS stimulation, mouse PXR-transfected cells were highly resistant to LPS-induced cytokine up-regulation (~10 fold). These data provide supporting evidence for establishing the existence of transcription factor crosstalk between the PXR and NF-κB in liver cells.

We next determined ligand-dependent or ligand-independent effect of mouse PXR on its activity. When mouse PXR was activated by PCN, the ligand-activated mouse PXR repressed LPS-induced gene expression at the comparable level to that of non-liganded mouse PXR. The results suggested that mouse PXR repressed NF-κB signaling pathway in a ligand-independent manner in Hepa1-6 cells.

The underlying molecular mechanism(s) responsible for the PXR repressive effect on inflammatory genes has been proposed. In response to LPS activation, PXR is SUMOylated by endogenous SUMO proteins at low stoichiometry. The SUMOylated PXR is highly dominant efficient to repress NF-κB transcriptional activity through protein-protein interactions, instead of direct DNA binding.

Figure 8

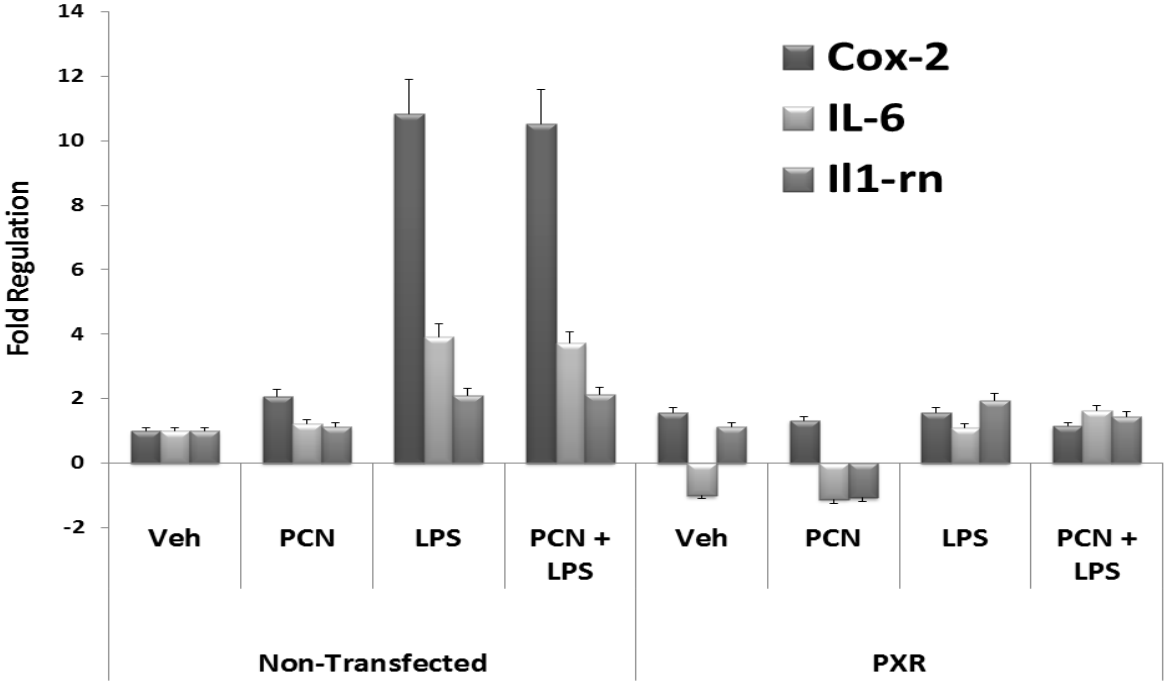


Figure 8. Expression of PXR Transrepresses the Induction of Endogenous Inflammatory Response Genes in Hepa1-6 Cells. Cells were transfected with mouse PXR or mock-transfected as indicated and treated with 10 mM PCN for 24 hours. Cells were treated for an additional 12 hours with LPS in the presence and absence of PCN as indicated. Gene expression levels of indicated endogenous inflammatory response genes were determined using real-time PCR as described in Materials and Methods. Data were expressed as the fold induction over the vehicle and were reported as the mean +/- SEM of three independent reactions.

2. The PXR Protein Is SUMOylated in vitro

To demonstrate that PXR is a substrate of SUMOylation, we first analyzed the PXR SUMOylation mechanism *in vitro*. There are 28 lysine residues on human PXR protein (Figure 9-1). Previously, the bioinformatic approach has been used to scan the amino acid sequence of human PXR for the presence of a consensus SUMOylation site (132). Using this strategy we identified four potential sites. Since the four sites are all located in the C-terminal hinge domain and LBD, the PXR protein was cut into two parts for the *in vitro* SUMOylation assay. The first part included AF-1 and DBD domains and was named as human PXR-DBD (1-105 amino acids). The second part included hinge domain, LBD and AF-2 domains and was referred as human PXR-LBD (106-434 amino acids).

The strategy shown in Figure 9-2 was followed to express, isolate and purify GST-tagged human PXR DBD and GST-tagged human PXR LBD proteins. Purified components were collected, separated on SDS-PAGE gels, and visualized by staining with Coomassie blue (Figure 9-3). As shown, the bands at 26kD in GST-human PXR DBD and LBD proteins lanes are de-associated GST proteins. The GST-human PXR LBD (64 kD) migrated slower than the GST-human PXR DBD (37kD) protein on the gel and we noticed that the higher molecular-weight protein was more degraded and less soluble in buffers.

We next used *in vitro* methods to determine the extent to which purified recombinant GST-human PXR LBD served as a substrate in the

SUMO-conjugation pathway. We incubated GST-tagged purified recombinant human PXR LBD *in vitro* together with E1, E2, E3, and SUMO-1 or SUMO-3 proteins in the presence or absence of the required magnesium and ATP co-factors. The extent of SUMOylation after the incubation was determined by SDS-PAGE and Western blot analysis with anti-hPXR monoclonal antibodies (Figure 9-4). This type of analysis revealed that the human PXR LBD protein can serve as an effective substrate for both SUMO-1 and SUMO-3 in the SUMO-conjugation pathway *in vitro*. And the *in vitro* SUMOylation of GST-human PXR LBD was strongly enhanced by E3 ligase PIASy. There is one primary SUMOylation band (~110kD) when either SUMO-1 or -3 was used in the reaction, which indicated human PXR LBD could be SUMOylated at one primary site *in vitro*. When we compared the molecular mass shift of the SUMO-1 modified band with SUMO-3 modified band on the blot, SUMO-1 conjugated PXR appeared at a higher molecular weight. And this is in consistent with the fact that SUMO-1 protein is larger in size than SUMO-3 protein. It is noteworthy that addition of PIASy dramatically enhanced SUMO modifications *in vitro*. In order to figure out whether GST-human PXR LBD could be SUMOylated without E3 *in vitro*, a blot with longer exposure time was shown in Figure 9-4 lower panel. The results suggested that GST-human PXR LBD was able to be SUMOylated to less extent by both SUMO-1 and SUMO-3 in the absence of E3 *in vitro*. The data presented indicated that SUMO-1 and SUMO-3 could modify GST-human PXR LBD *in vitro,* and SUMOylation was enhanced by PIASy. Although the *in vitro* assay is highly suggestive of potential PXR

SUMO modification, it is still necessary to examine PXR SUMO conjugation in cultured cell lines or primary cells.

Figure 9-1

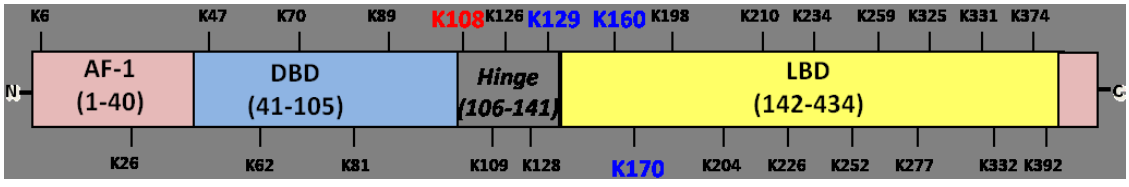


Figure 9-1. Structure and Post-translational Modification of Human PXR Lysine Residues. **A)** All the lysine residues on human PXR were determined and there are 28 lysine residues. The human PXR protein was analyzed for the presence of the consensus SUMOylation sequences as defined by an online SUMO Plot server (http://www.abgent.com/tools/SUMOplot). This type of bioinformatic analysis identifies four potential sites for SUMOylation, one of which is predicted as a "high probability" SUMOylation site and three others that are predicted as "low probability" SUMOylation sites.

Figure 9-2

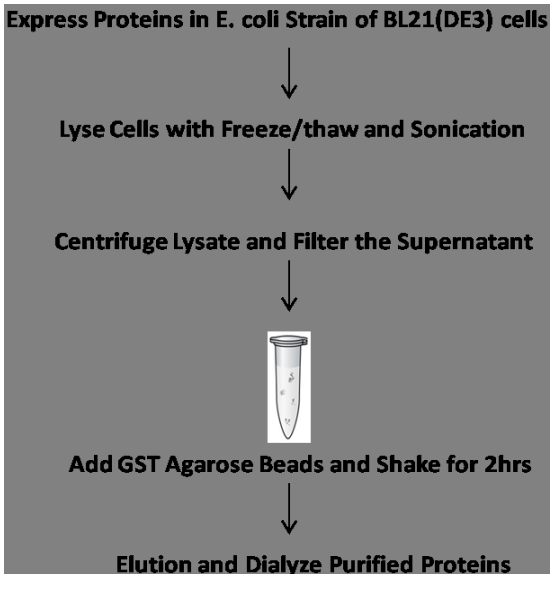
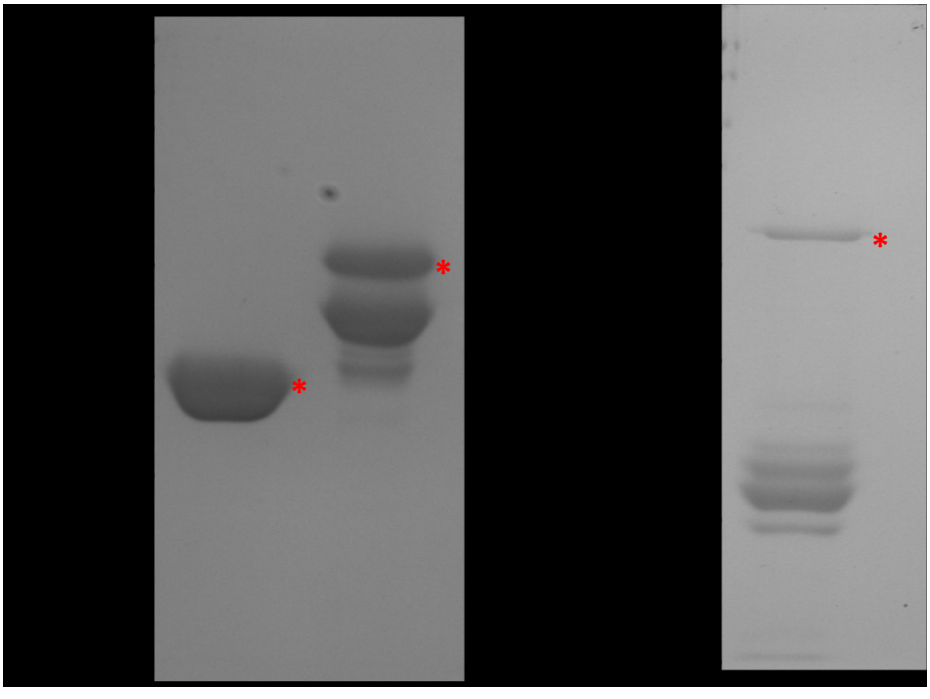


Figure 9-2. Purification Scheme for GST-tagged Proteins.

Figure 9-3

A) **B)**



GST **GST-human PXR DBD** **GST-human PXR LBD**

Figure 9-3. SDS-PAGE Analysis of Purified Proteins. A) GST and GST-human PXR DBD proteins were separated on a 12.5% gel. **B)** GST-human PXR LBD protein was separated on a 10% gel. Proteins were visualized by staining with Coomassie blue. The purified proteins are indicated with asterisks.

Figure 9-4

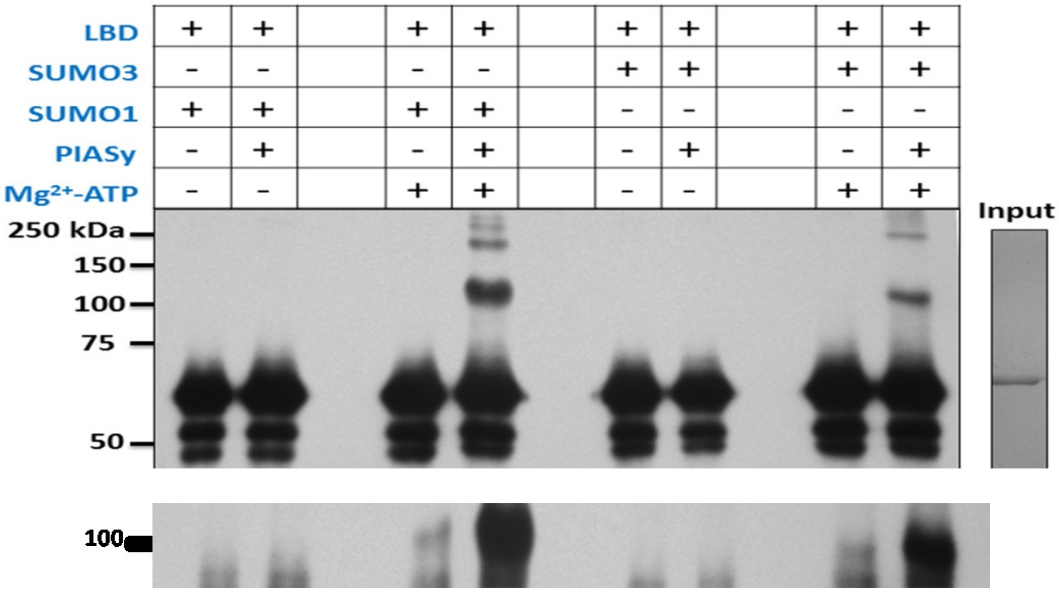


Figure 9-4. *In Vitro* SUMOylation of GST-human PXR LBD by SUMO-1 and SUMO-3 is enhanced by PIASy. The recombinant purified GST-human PXR LBD fusion protein was incubated together with E1, E2 in the presence and absence of Mg^{2+}-ATP as indicated. The recombinant purified E3 ligase (PIASy) was included as indicated. Input protein Coomassie stained gel is shown in the inset at the right (Input). Five microliter aliquots of the *in vitro* SUMOylation reaction were resolved using 10% SDS-PAGE and western blotting was performed using anti-human PXR monoclonal antibodies to detect SUMO-modification. The long-exposure blot is shown at the lower panel.

3. PXR is Preferentially SUMOylated in Hepa1-6 Cells by SUMO-3 and PIASy.

We initiated a series of studies using an over-expression and transfection approach in Hepa1-6 and HeLa cells (132). Co-transfection of Hepa1-6 cells with 1) the human PXR expression vector, 2) the 6×His-SUMO-1 or 6×His-SUMO-3 expression vector and 3) the PIAS1 or PIASy E3 enzyme expression vector allows the rapid and selective purification of SUMOylated forms of PXR using cobalt-linked agarose beads and a strong denaturing buffer containing high levels of guanidine-HCl. The reason for choosing cobalt beads instead of nickel beads is that cobalt beads exert stronger and more specific binding to SUMOylated human PXR (data not shown). But the human PXR might still non-specifically bind to cobalt beads through zinc fingers.

Hepa1-6 cells have endogenous E1 and E2 enzymes which can support PXR SUMO modification efficiently without over-expression of E1 and E2. The endogenous SENPs that would probably cleave SUMOylated forms of PXR during cell lysis are rapidly de-activated under denaturing conditions.

Based on this experimental approach we detected SUMOylated PXR using a Western blot analysis with the α-human PXR monoclonal antibody (Figure 10-1). When human PXR alone was exogenously expressed, the un-conjugated human PXR (~52kD) was pulled down by cobalt-linked beads which represented the non-specific binding. When His-tagged SUMO plasmid and E3 ligase PIAS were co-transfected, the un-conjugated human PXR was detected along with a

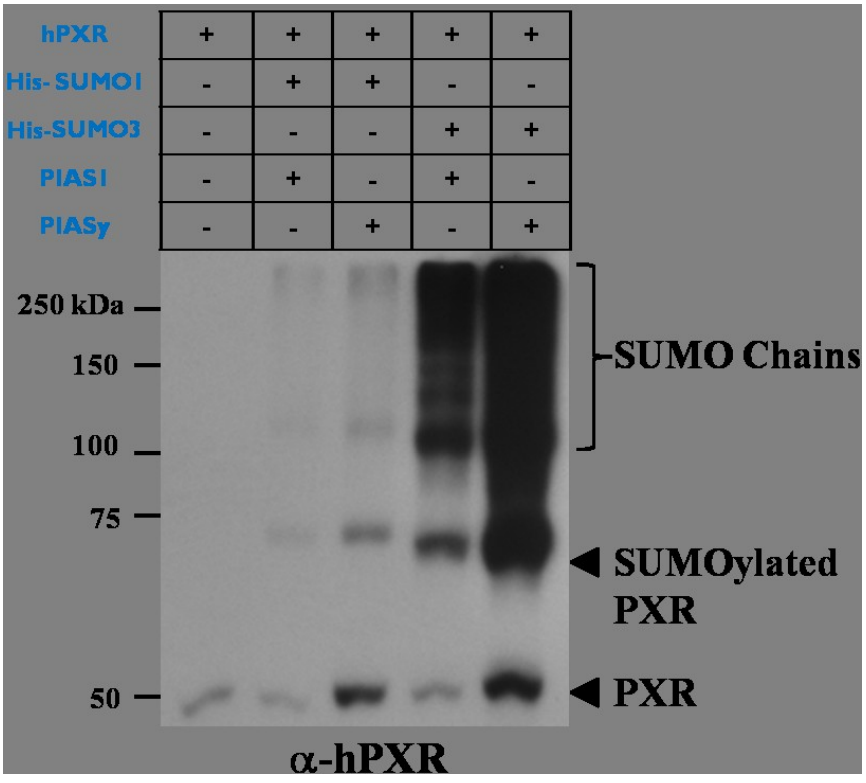
series of higher molecular weight proteins that represented SUMO-conjugated human PXR.

The over-expressed SUMO-3 protein formed chains on the human PXR protein, which were present at a much higher level in cells transfected with PIASy compared with those transfected with PIAS1(Figure 10-1 lane 4 and 5). We also used SUMO-2/3 antibody to detect global SUMO-2/3 modification (Figure 10-2). PIASy preferentially increased global SUMO modification by SUMO-2/3 to a higher level compared with PIAS1. The whole cell lysates of Hepa1-6 cells were subjected to detection of endogenous SUMO-2/3 proteins. Consistent with the prevailing theory that SUMO-2/3 remains in a free or un-conjugated form under normal condition (178), the Hepa1-6 whole cell lysates showed endogenous free and un-conjugated SUMO-2/3 proteins (Figure 10-3 lower panel).

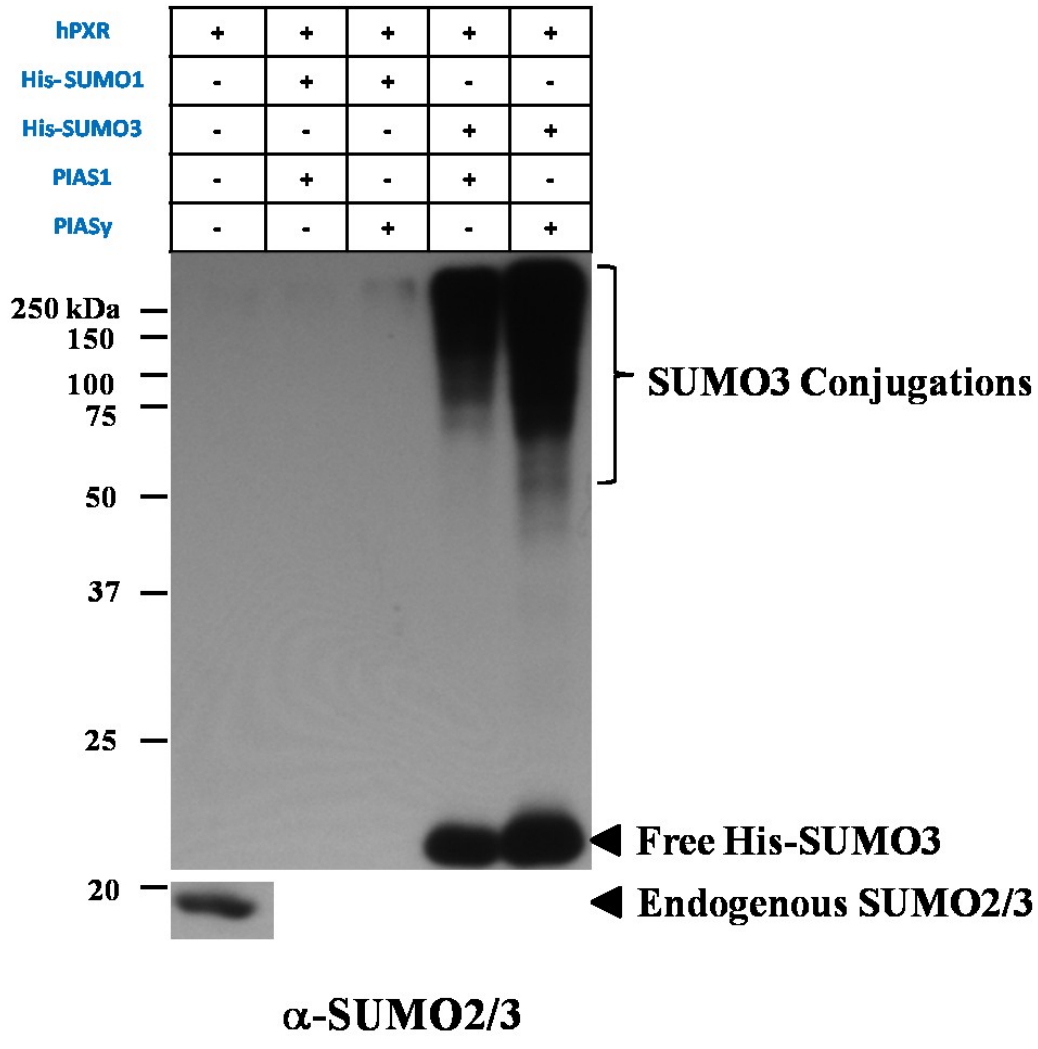
PXR could also be SUMOylated by SUMO-1 in our cell-based assay, although we found that human PXR was preferentially modified by SUMO-3. SUMO-1 cannot form chains on PXR since its self did not possess consensus SUMO sequences. PIASy preferentially promoted SUMO-1 modification both on human PXR and global proteins as shown in Figure 10-4. When the blot was over-exposed, the free His-tagged SUMO-1 was shown to be present (Figure 10-4 middle panel). Besides endogenous SUMO-2/3, the Hepa1-6 cells contained endogenous SUMO-1 protein as well (Figure 10-1-C lower panel).

Figure 10-1

A)


hPXR + + + + +
His-SUMO1 - + + - -
His-SUMO3 - - - + +
PIAS1 - + - + -
PIASy - - + - +
250 kDa
150
100
75
50
SUMO Chains
SUMOylated PXR
PXR
α-hPXR

B)


hPXR + + + + +
His-SUMO1 - + + - -
His-SUMO3 - - - + +
PIAS1 - + - + -
PIASγ - - + - +
250 kDa
150
100
75
50
37
25
20
SUMO3 Conjugations
Free His-SUMO3
Endogenous SUMO2/3
α-SUMO2/3

C)

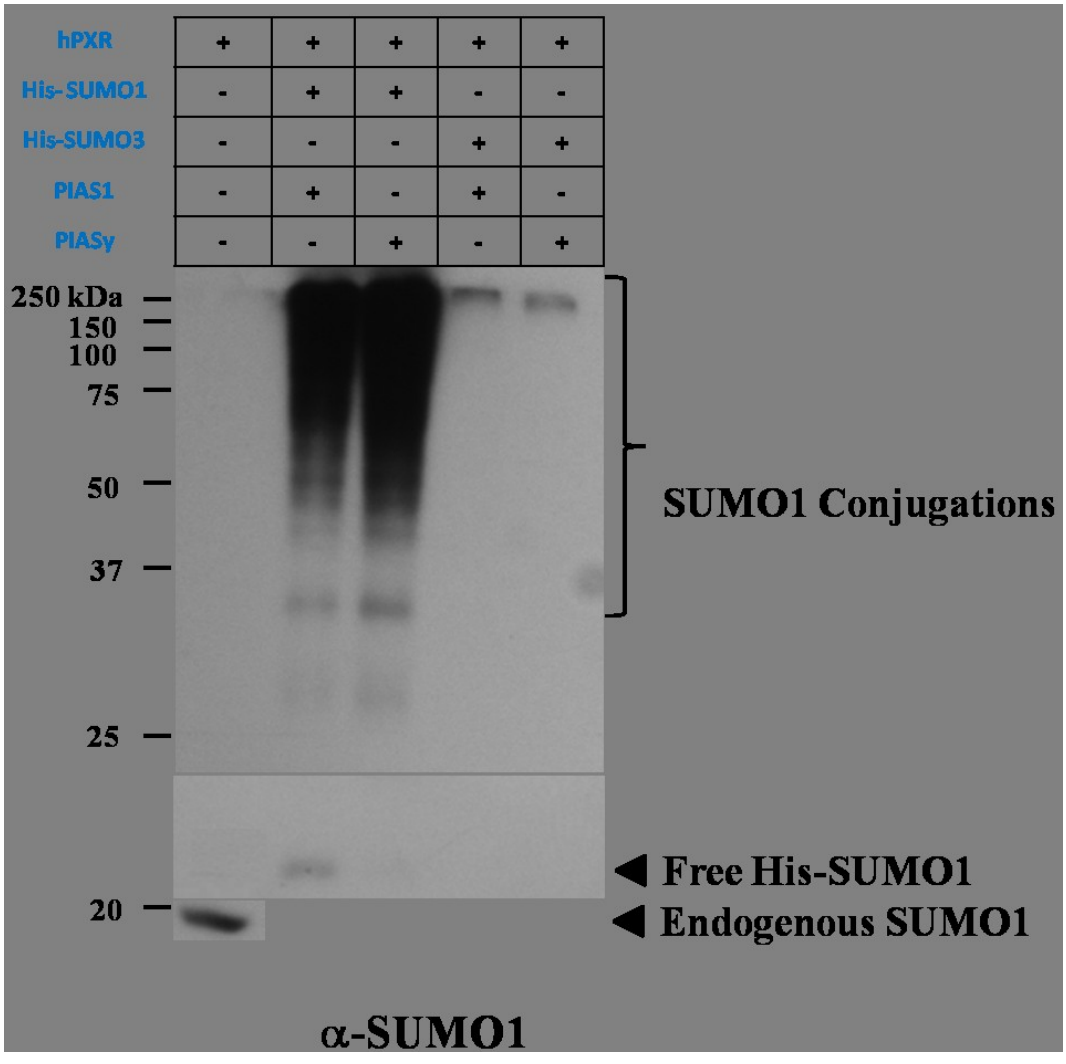


Figure 10-1. Detection of SUMOylated Human PXR Protein in Hepa1-6 Cells. The human PXR protein was co-expressed in Hepa1-6 cells with 6X-His-tagged SUMO-1 or SUMO-3, and E3 enzyme (PIAS1 or PIASy). After 48h transfection, cells were lysed using denaturing buffer containing guanidinium hydrochloride to inactivate de-SUMOylation enzymes. SUMOylated proteins were purified by cobalt-linked agarose beads. Purified His-tagged proteins and Hepa1-6 whole cell lysates were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize A) human PXR or B) SUMO-2/3 or C) SUMO-1 as indicated.

4. The hPXR-K108 Residue Is A SUMOylation Site.

It was previously reported that human PXR contains multiple SUMO consensus sequences and that one site, K108, is of high probability (Figure 9-1). To directly assess K108-SUMO conjugation capacity, the PXR protein containing site108 lysine-to-arginine mutation was employed. We used cell-based assay to detect the extent of human PXR-K108R SUMO-1 and SUMO-3 modification (Figure 11). The K108R mutation down-regulated, but did not blocked the SUMOylation of PXR. The result indicated that K108 was involved in both SUMO-1 and SUMO-3 conjugation pathway. The monomeric SUMO modified-PXR still existed which suggested there were other potential SUMOylation sites besides K108, which calls for further analysis. Another thing to note is that endogenous SUMO-2/3 proteins may contribute to unexpected migrating form of human PXR detected in SUMO-1 over-expression groups because the endogenous free-state SUMO-2/3 in Hepa1-6 cells is likely to be conjugated to human PXR when PIASy is over-expressed.

5. Inflammatory Mediator LPS Induces SUMOylation of Human PXR Wide Type and K108R Mutant.

We next sought to determine if inflammation could influence SUMOylation of PXR in Hepa1-6 cells. Previous results performed in human hepatocytes reported that treatment of cells with TNFα alone or TNFα together with Rif produced an increased level of SUMO-2/3 modified PXR (132). The identification of PXR SUMO(1)ylation raised the question of whether LPS also induced SUMO-1 conjugation to PXR.

The Hepa1-6 cells were transfected with plasmids that express human PXR, His-tagged SUMO-1 and E3 PIASy. Transfected cultures were subjected to vehicle treatment (Figure 12-1, lane1) or LPS treatment for 12 hours (Figure 12-1, lane 3). SUMO-modified proteins were purified after drug treatment using cobalt bound beads and PXR protein was examined on Western blots. As expected, LPS-treated cells significantly induced SUMO(1)ylation of human PXR at 12 hrs (Figure 12-1, lane 3).

Some NR ligands enhance SUMOylation of target NR family members (e.g., LXRα and PPARγ), but this has not been detected for PXR. Transfected Hepa1-6 cells were treated with either vehicle (DMSO) (Figure 12-1, lane 1) or the human PXR ligand Rif (Figure 12-1, lane 2) for 36 hours before harvest. Rif treatment significantly decreased SUMO(1)ylation of human PXR compared with vehicle (Figure 12-1, lane 1). Next we compared ligand treatment group with co-treatment group comprising of both ligand pre-activation and inflammatory stimulation (Figure 12-1, lane 4). LPS co-treatment ameliorated ligand-induced down-regulation of PXR SUMO1 modification. These results indicated that inflammatory signaling induced both non-liganded and ligand-bound PXR SUMO-1 modification.

Since K108 is one of the potential SUMO acceptor sites, we next analyzed human PXR K108R SUMO-1 conjugation levels in response to LPS stimulation. As shown in Figure 12-2 left panel, LPS induced SUMO-1 modification on human PXR K108R mutant at a comparable level to that of wide type human PXR. The global SUMO-1 conjugations in response to LPS did not distinguish between over-expressed wide type and mutated human PXR groups. The data

revealed a tentative result that K108 was not required for LPS-inducible PXR SUMOylation.

Figure 11

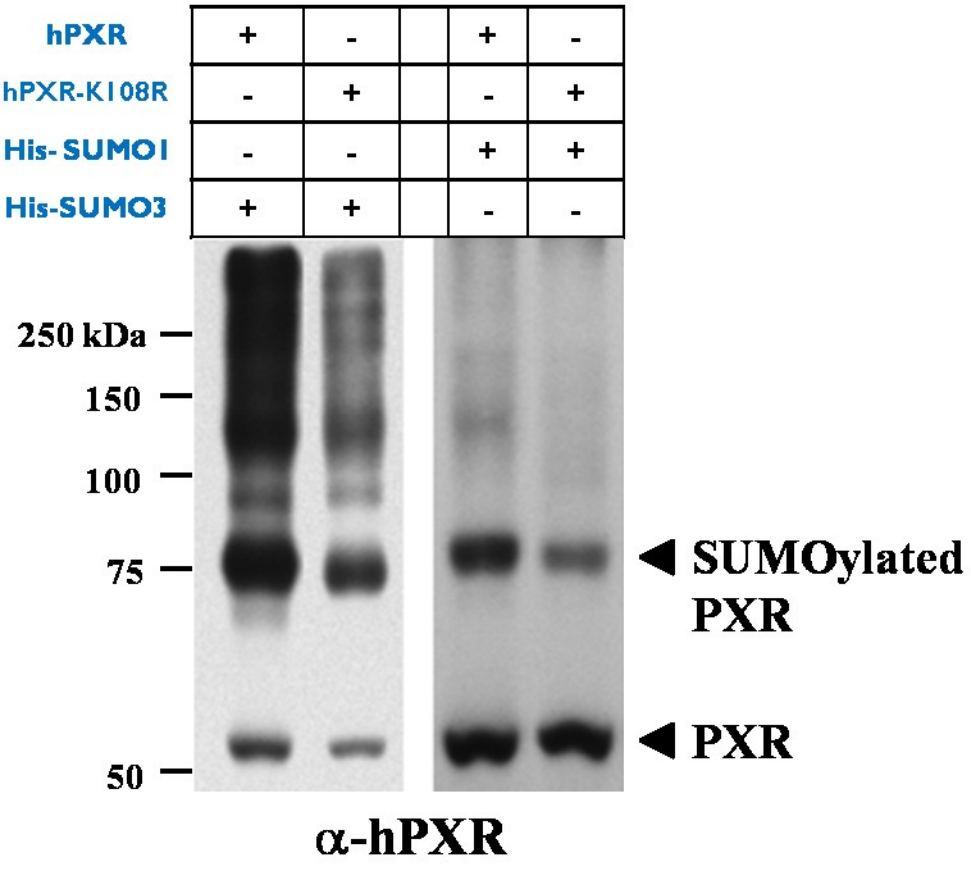


Figure 11. The Lysine Residue 108 Mutation Decreases PXR SUMOylation. Hepa1-6 cells were transfected with His-tagged SUMO1 or SUMO3 and either human PXR or human PXR-K108R. The ~75kDa bands were SUMOylated PXR and were reduced in human PXR-K108R SUMO-1 and SUMO-3 conjugation groups.

Figure 12-1

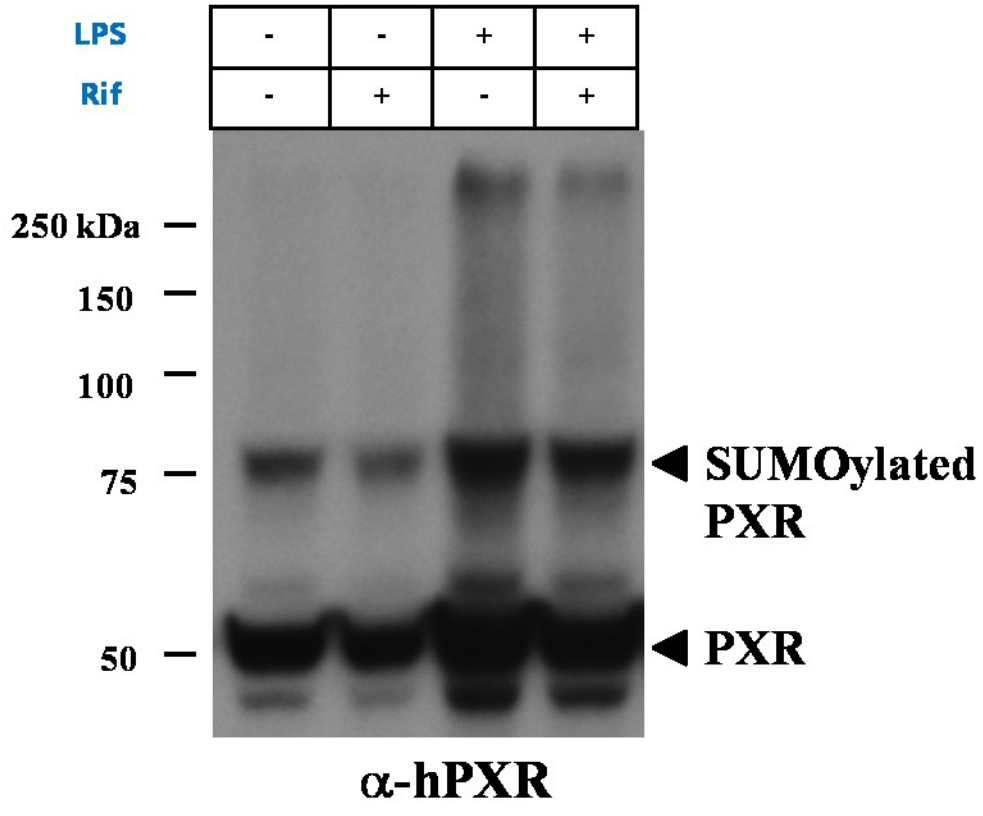


Figure 12-1. Inflammatory Stimulus and Ligand Modulate Wide Type Human PXR Protein SUMO-1 Modification in Hepa1-6 Cells. Hepa1-6 cells were transfected with human PXR, PIASy and His-tagged SUMO1, or SUMO-3. Cells were treated for 36 hours with vehicle or Rif (10 μM) or LPS (10 mg/ml) for 12 hours, or Rif (10 μM) pre-treatment together with LPS (10 mg/ml) 12 hours co-treatment. The cells were harvested 48hrs post-transfection and purified proteins were immunoblotted with anti-human PXR.

Figure 12-2

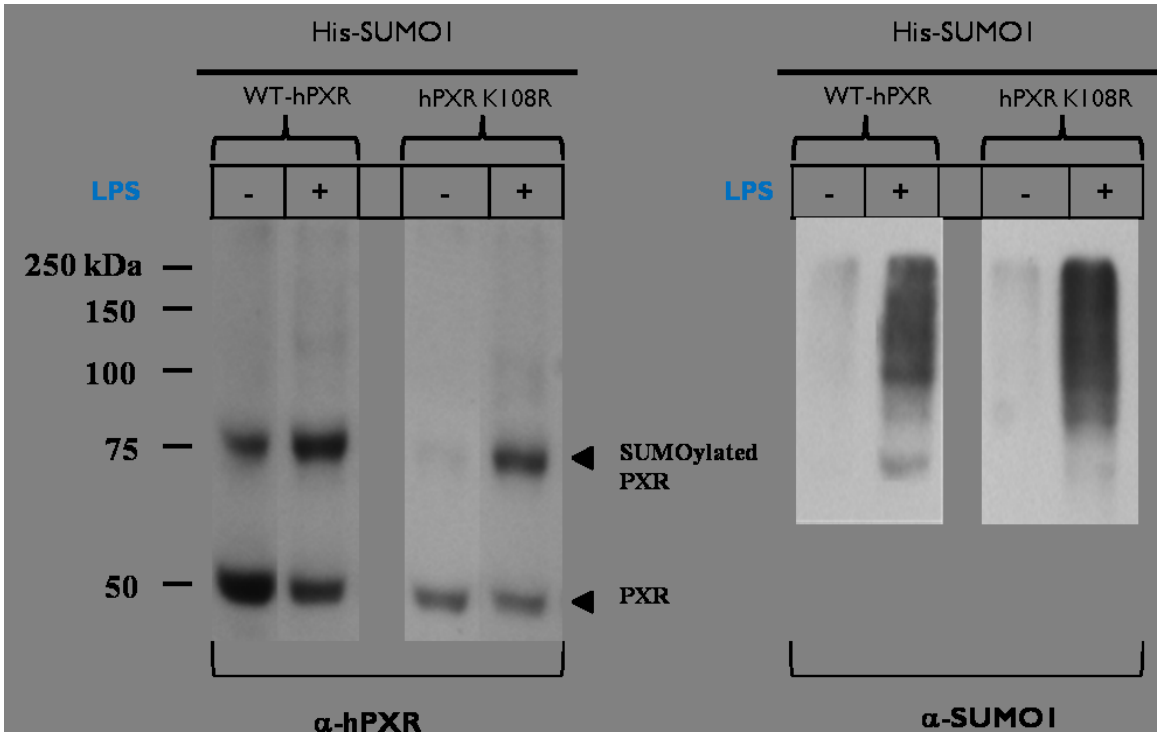


Figure 12-2. K108 Is Not the Primary LPS-inducible SUMOylation residue. Hepa1-6 cells were transfected with SUMO-1 and either human PXR or human PXR-K108R and treated with vehicle or LPS (10 mg/ml) for 12hrs before harvest. The purified protein extracts were analyzed by immunoblotting with PXR monoclonal antibody and anti-SUMO1 antibody.

6. Sentrin Proteases Differentially De-SUMOylate PXR-SUMO Conjugates in Hepa1-6 Cells.

Because SUMOylation is a reversible pathway, we next studied the reversibility of PXR SUMOylation reaction and how SUMOylated PXR may be selectively processed by six distinct mammalian SUMO proteases.

We first examined the capability of SENP1, -2, -3, -5, -6 and -7 to de-conjugate SUMO-1 from PXR. All SENPs were expressed as N-terminal Flag fusions, and Hepa1-6 cells were transfected with expression plasmids encoding His-tagged SUMO-1 and PIASy. Because His-tagged SUMO-1 was expressed as the C-terminally processed Gly-Gly form, our de-conjugation assays in Hepa1-6 cells compared merely the isopeptidase activity of different SENPs. As shown in Fig. 13-1, SENP1 and SENP2 were potent in removing SUMO-1 from human PXR protein. The isopeptidase function of SENP3 was very weak on SUMO-1-modified human PXR. SENP5, 6 and 7 showed no activity on SUMO-1 conjugates (Figure 13-1).

We next compared the isopeptidase activity of the SENPs toward human PXR-SUMO-3 conjugations in Hepa1-6 cells. In line with the above results for human PXR-SUMO-1 conjugates de-SUMOylation, both SENP1 and SENP2 were efficient in de-conjugating SUMO-3 from human PXR in Hepa1-6 cells. Interestingly, SENP3 and 5 were also capable of de-conjugating SUMO-3 to some extent. Thus, SENP3 and SENP5 appeared to show preference for SUMO-3 conjugates. SENP6, which showed no activity on human PXR-SUMO1 modifications in Hepa1-6 cells, was efficient toward human

PXR-SUMO3 conjugates. It completely removed SUMO3 chain formation. SENP7 was characterized as a SUMO-2/3-specific protease that was likely to regulate poly-SUMO-2/3chains rather than SUMO-1 conjugation (176). However, SENP7 had no effect on removing SUMO-3 from the hPXR–SUMO3 conjugates (Figure 13-2).

Mutation of the catalytic cysteine in the C-terminal of SENPs to serine has been shown to inactivate their catalytic function (179). We used the mutants SENP1C603S, SENP2C466S, SENP3C532S, SENP5C713S, and SENP6C1030S as controls in Hepa1-6 cells de-SUMOylation assays. In keeping with the importance of the conserved cysteine residue for the fully catalytic function of SENPs, the mutated SENPs were totally inactive in de-conjugating SUMOs from cellular proteins (Fig. 13-1 and 13-2).

Taken together, these results identified SENP1 and SENP2 as strong candidates for SUMO-specific proteases reversing PXR SUMOylation by both SUMO-1 and SUMO-3 in cells. SENP3 and 5 showed substrate preferences towards SUMO-3 conjugates. SENP6 effectively removed SUMO-3 poly-chain formation. SENP7 had no effect on PXR SUMOylation.

Figure 13-1

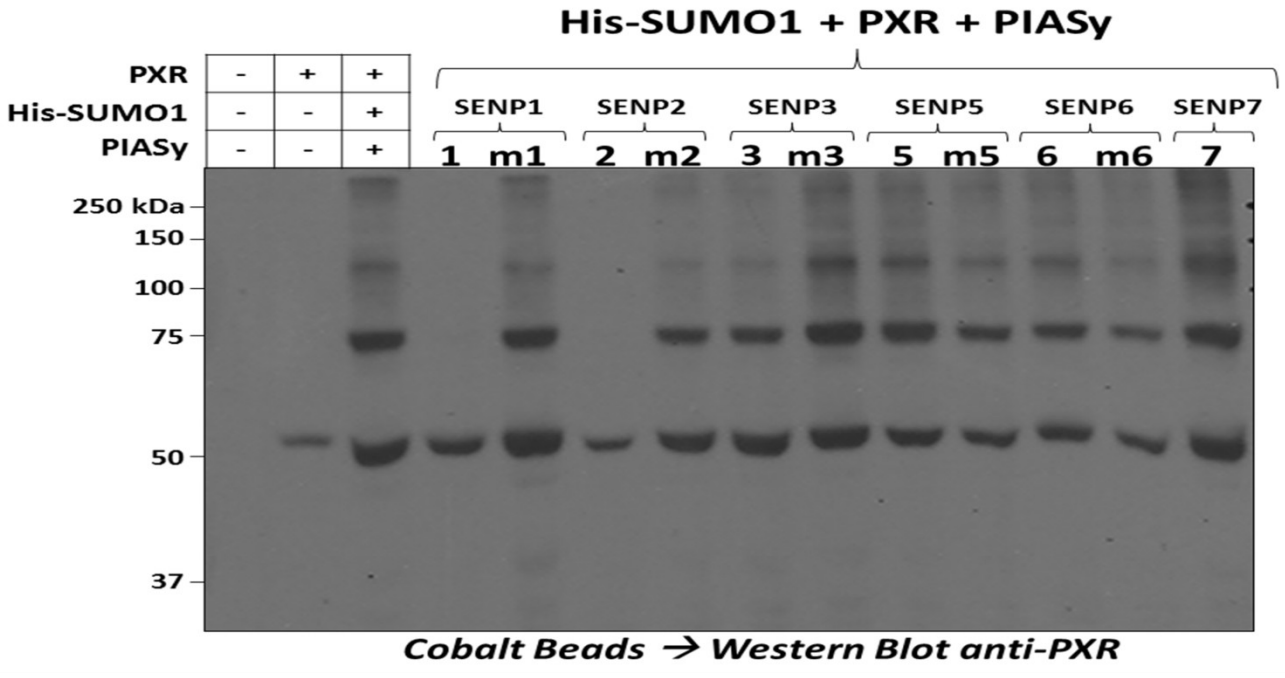


Figure 13-1. Cell-based Analysis of Human PXR SUMOylation/De-SUMOylation by SUMO1 and Sentrin Proteases in Hepa1-6 Cells. Indicated mammalian expression vectors are transfected using lipofectamine per manufacturer's instructions. Forty-eight hrs post-transfection cells were lysed as described in Materials and Methods. Proteins were resolved using 10% SDS-PAGE and western blotting was performed using anti-human PXR monoclonal antibody. m1:SENP1C603S, m2: SENP2C466S, m3: SENP3C532S, m5: SENP5C713S, m6: SENP6C1030S

Figure 13-2

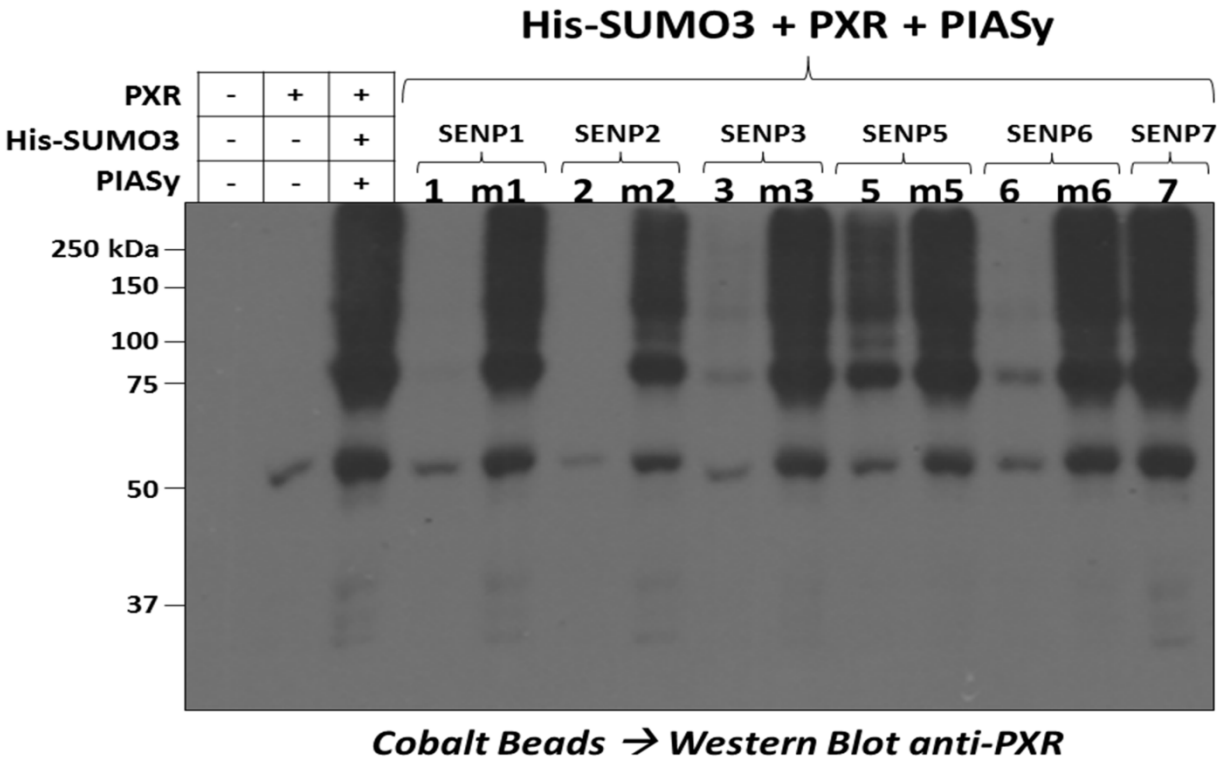


Figure 13-2. Cell-based Analysis of Human PXR SUMOylation/De-SUMOylation by SUMO-3 and Sentrin Proteases in Hepa1-6 Cells. Indicated mammalian expression vectors are transfected as described. Proteins were resolved using 10% SDS-PAGE and western blotting was performed using anti-human PXR monoclonal antibody.

7. SENP6 together with Human PXR Mutants Provide Insights into PXR SUMOylation Sites Prediction

Taken together, the results shown above suggest that 1) The ~75kD band is the primary human PXR SUMOylation band, 2) K108 is one of the potential SUMOylation sites, 3) The ~110 kD band indicates that there are two SUMO proteins conjugated to PXR. We hypothesized that there were two major SUMOylation sites. As discussed above, Hepa1-6 cells have high amount of endogenous SUMO-2/3 proteins which may lead to chain formation.

In order to further map PXR SUMOylation sites, human PXR-K108R was transfected together with SENP6 to remove all possible SUMO chain formation. As shown in Figure 14-1, human PXR-K108R mutation together with SENP6 isopeptidase left two monomeric bands (~75 kD and ~110kD). The data suggest that there could be at least three SUMO-3 modifiable sites on human PXR (Figure 14-1).

When we looked at SUMO-1 modification, we found that SENP6 cleaved the higher molecular bands on hPXR-K108R and left one primary monomeric SUMO-1 site (~75kD). We speculated that two major sites were modified by SUMO-1 (Figure 14-2).

The double mutants and triple mutants were used to further analyze the potential SUMOylation sites. The systematic site-directed mutagenesis approach revealed two major sites of SUMOylation at positions K108 and K128 on the human PXR protein. The K108R, K128R double mutant could not be modified

by SUMO-1 (Fig 14-3 lane 7); whereas it could still form monomeric conjugates by SUMO-3 (Fig 14-4 lane7) (~75kD).

Figure 14-1

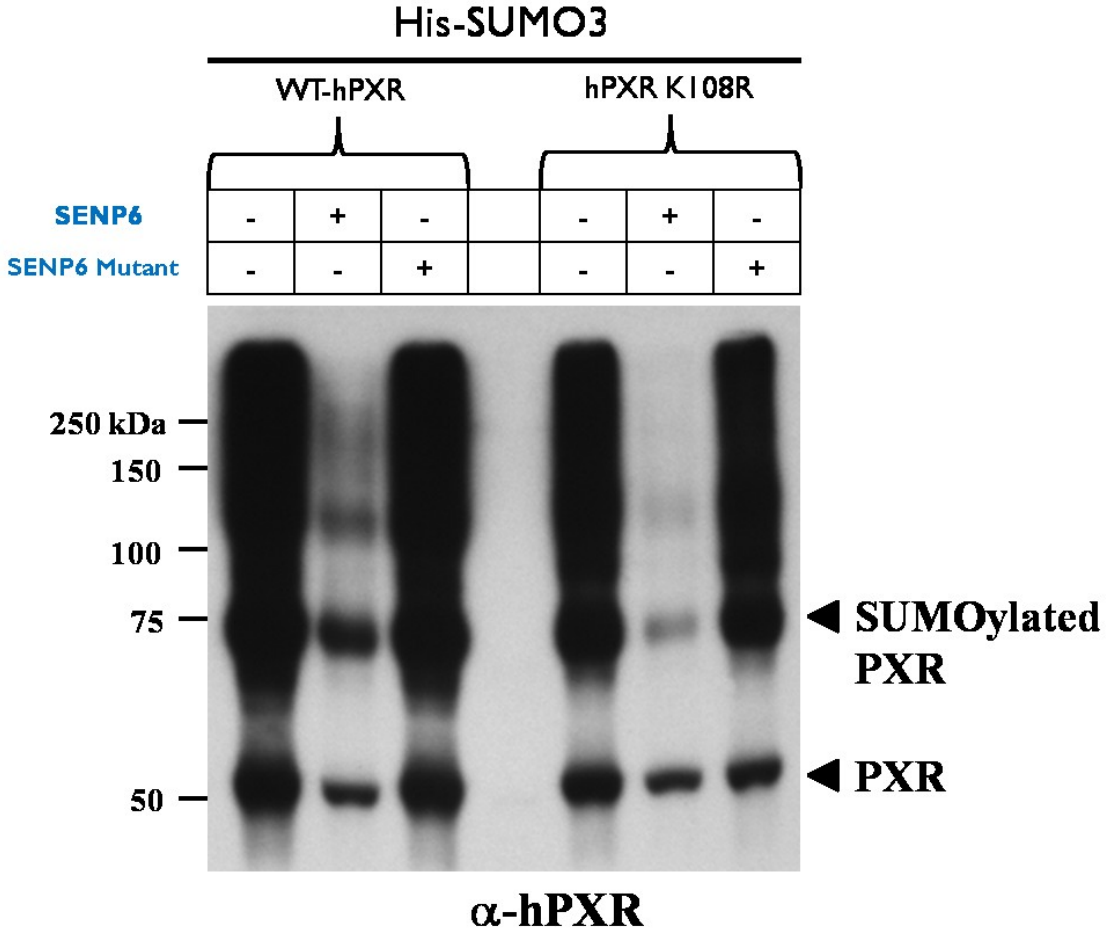


Figure 14-1. Detection Wide Type Human PXR and Human PXR-K108R Mutant SUMO-3 Modification in Hepa1-6 Cells. The human PXR protein or hPXR-K108R was co-expressed in Hepa1-6 cells together with His-tagged SUMO3 proteins, E3 enzyme PIASy and SENP6. After 48h transfection, cells were lysed and SUMOylated proteins were purified by using cobalt-linked agarose beads. Purified proteins were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize human PXR as indicated.

Figure 14-2

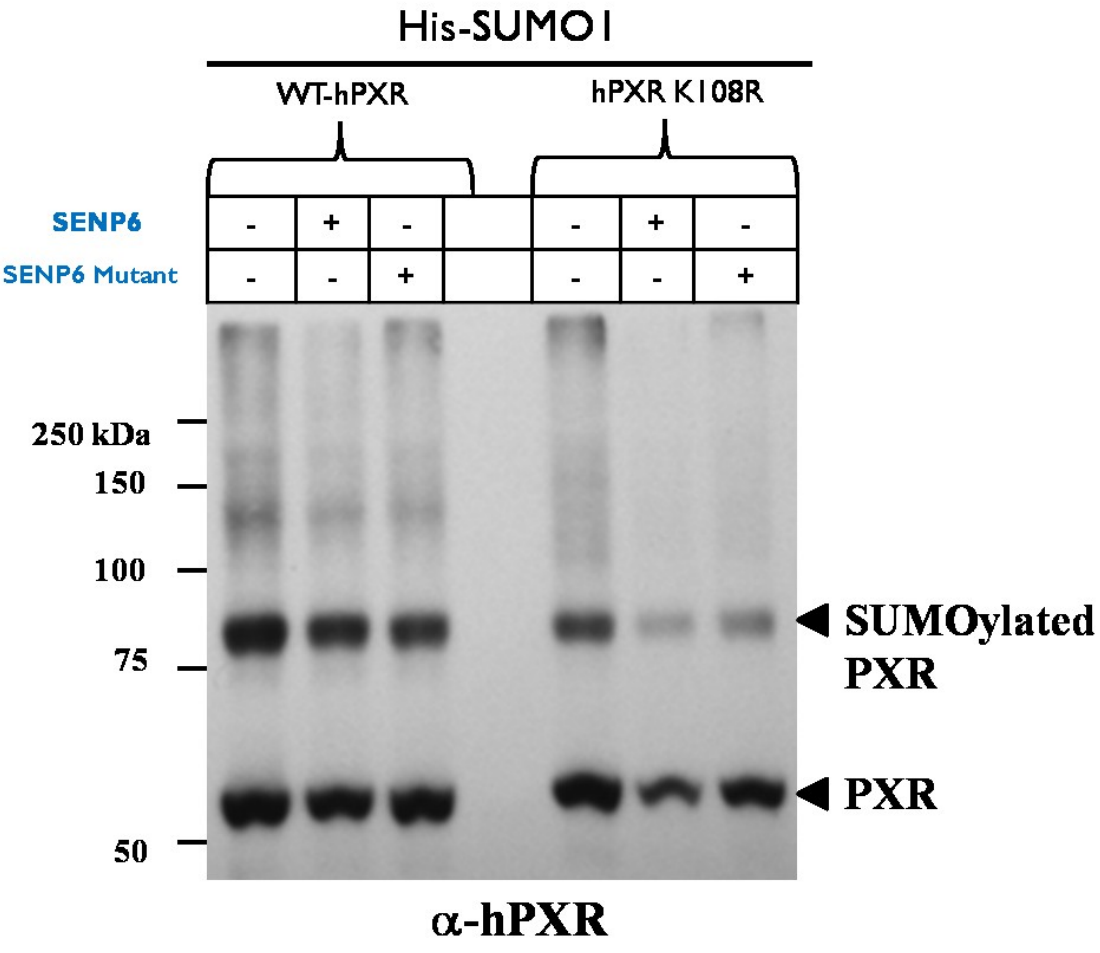


Figure 14-2. Detection Wide Type Human PXR and Human PXR-K108R Mutant SUMO-1 Modification in Hepa1-6 Cells. The human PXR protein or hPXR-K108R was co-expressed in Hepa1-6 cells together with His-tagged SUMO1 proteins, E3 enzyme PIASy and SENP6. After 48h transfection, cells were lysed and SUMOylated proteins were purified by using cobalt-linked agarose beads. Purified proteins were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize human PXR as indicated.

Figure 14-3

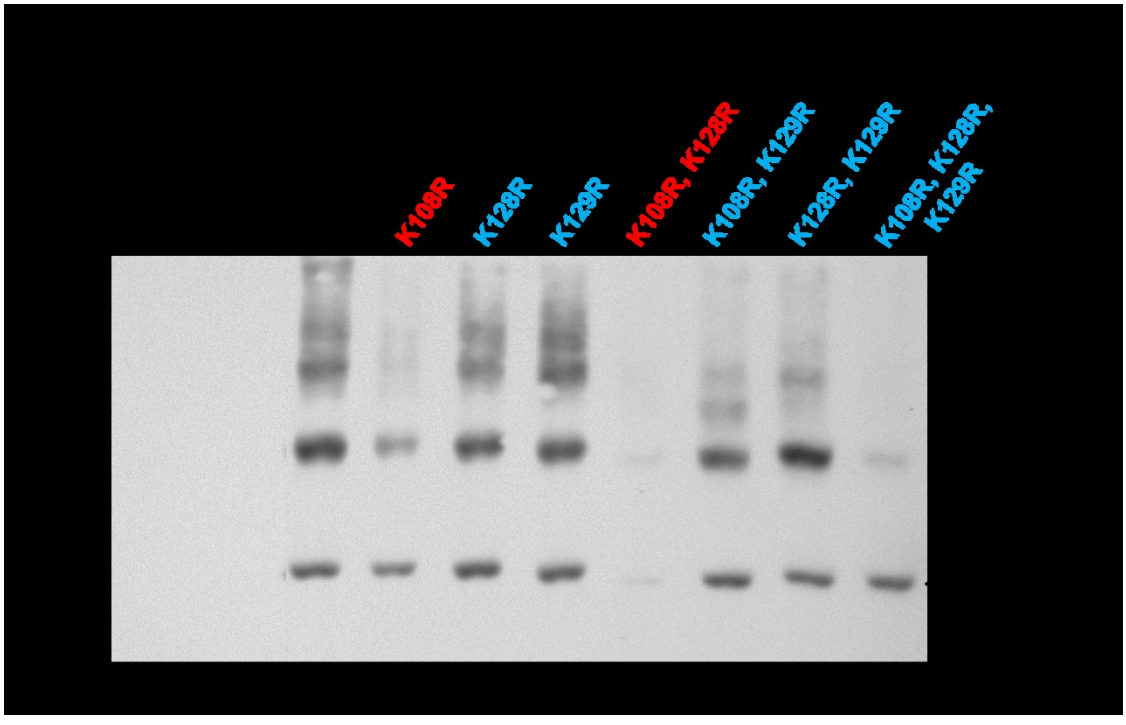


Figure 14-3. PXR is Mainly SUMOylated in the Hinge Domain by SUMO-1. The human PXR protein and seven human PXR mutants were expressed in Hepa1-6 cells together with His-tagged SUMO1 proteins and E3 enzyme PIASy. After 48h transfection, cells were lysed and SUMOylated proteins were purified by using cobalt-linked agarose beads. Purified proteins were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize human PXR as indicated.

Figure 14-4

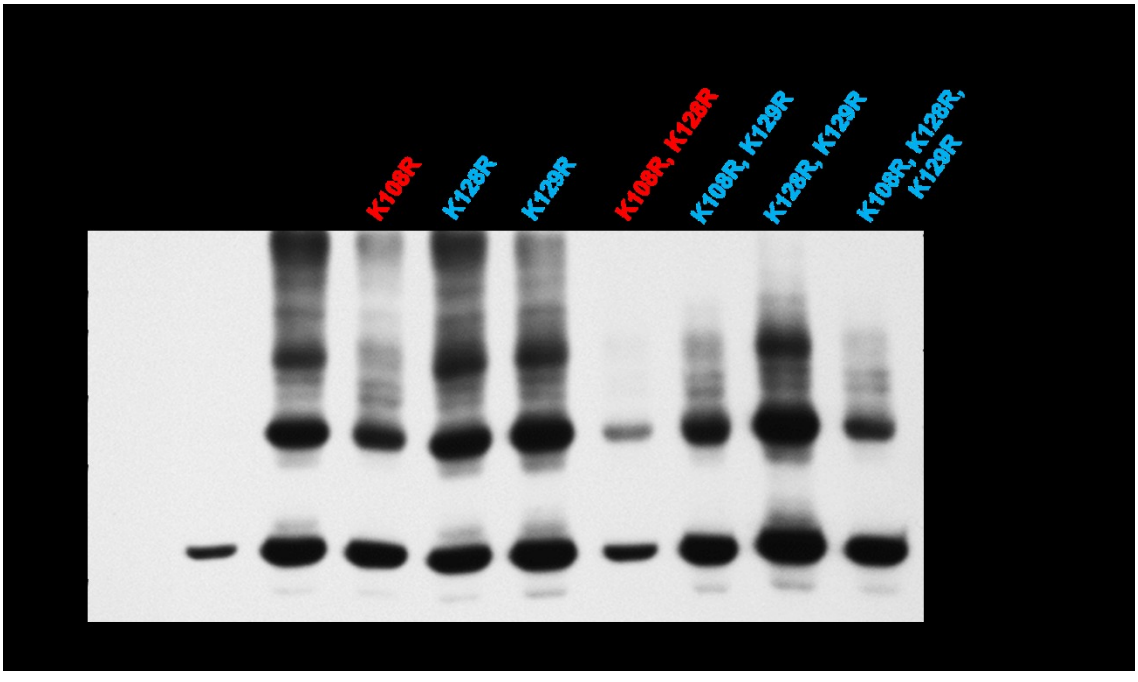


Figure 14-4. PXR is Mainly SUMOylated in the Hinge Domain by SUMO-3. The human PXR protein and seven human PXR mutants were expressed in Hepa1-6 cells together with His-tagged SUMO3 proteins and E3 enzyme PIASy. After 48h transfection, cells were lysed and SUMOylated proteins were purified by using cobalt-linked agarose beads. Purified proteins were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize human PXR as indicated.

Discussion

1. PXR SUMOylation/De-SUMOylation Mechanism

1.1 SUMO-1 versus SUMO-2/3

The PXR protein belongs to a family of ligand-activated transcription factors that regulate xenobiotic response and drug metabolism in liver and intestine. Recent studies have characterized PXR as a regulator of hepatic innate and adaptive immune response. PXR agonist Rif exerts immunological effect (122) and PXR is required for normal immunity to bacterial infection (180). In addition, PXR activation inhibits LPS induction of a number of pro-inflammatory genes (132). Repression of inflammatory mediator by PXR agonists is abolished in hepatocytes lacking PXR indicating that PXR mediates this anti-inflammatory response (181).

Although recent results have provided a link between xenobiotic metabolism and inflammation, relatively few mechanisms have been established for the transrepression pathway regarding the post-translational modification of the human PXR protein. Our lab has previously identified that PXR SUMOylation is implicated to repress inflammatory response in hepatocytes (132).

Here we provided direct evidence of PXR modification by both SUMO-1 and SUMO-2/3. Previous study showed the existence of endogenous un-conjugated form of SUMO-2/3 in great abundance and the free state SUMO-1 to a lesser extent in COS-7 cells (178). In our mouse hepatoma cell

model, both SUMO-1 and SUMO-2/3 are endogenously expressed in the un-conjugated form with the potential capacity to modify cellular proteins. Over-expression of SUMO proteins and E3 ligases markedly increased the level of human PXR modification by both SUMO-1 and SUMO-3. Furthermore, compared with SUMO-1, PXR was preferentially targeted by SUMO-3 which formed poly-SUMO chains *in vivo*.

In vitro SUMOylation assays showed that PXR LBD was a substrate for both SUMO-1 and SUMO-3. PXR LBD was preferentially targeted by SUMO-1 rather than SUMO-3 *in vitro* which contradictory to the cell-based result. However, it is questionable whether the *in vitro* SUMOylation assays could faithfully reproduce physiological substrate selection mechanisms.

The endogenous free and un-conjugated SUMO-2/3 is readily available for conjugation reactions when a series of stress-responsive kinase cascades is activated in response to cellular stresses such as heat shock (178), hypoxia/ischemia (182) and oxidative stress (183), whereas SUMO-1 modification level appears to be unaffected (184). The increase of incorporation of SUMO-2/3 into PXR and formation of SUMO-2/3 chains were found in response to TNFα stimulation and the feedback mediated active repression of NF-κB activity in hepatocytes (132). Here, we found that SUMO-1 modification of PXR was also induced in LPS-mediated inflammatory response in Hepa1-6 cells. On the contrary, ligand activation was observed to lead to a reduction in the amount of SUMO-1 conjugation to PXR. Such changes in modification have been observed by other NRs as well, for example PPARγ2

(185) and VDR (186). Ligand binding might induce PXR conformation changes which results in reduced accessibility of acceptor sites to SUMO modification.

Since we only utilized Hepa1-6 cell line in our studies, future work on detecting SUMO-1 modification of endogenous human PXR in primary cultures in response to inflammatory stimulation is necessary. If inflammation actively induced endogenous PXR SUMO-1 conjugation in human hepatocytes, we could further focus on a comparison of functional consequences between SUMO-1 conjugation and SUMO-3 conjugation. It is possible that SUMO-1- and SUMO-3-dependent transrepression pathways converge on the regulation of NCoR clearance from inflammatory response genes. It will be of interest to study the signal-specific and gene-specific usage of the two parallel pathways by PXR. Also characterization of proteins specifically modified by SUMO-2/3 or SUMO-1 in a stress-responsive manner and the fate of such SUMO-modified proteins is an interesting question to study.

It has been shown that SUMO-2/3 possesses the capacity to form polymeric chains via lysine 11. Interestingly, SUMO-1 has been found to be conjugated to lysine 11 on SUMO-2/3, suggesting that SUMO-1 may act as a SUMO-2/3 chain terminator *in vivo* (187). The physiological significance of these polymeric forms of SUMO-2/3 requires further discussion. Intriguingly, it has been reported that ubiquitination was induced in response to a variety of cellular damages and environmental stresses such as heat shock, oxidative stress, viral infection, or mutation (188,189), which results in rapid degradation of damaged cellular proteins by 26S proteasomes. Because many proteins are substrates for both SUMO and ubiquitin, it has been suggested that SUMO might compete

with ubiquitin for the same lysine residue and stabilize the target protein by preventing ubiquitin-mediated proteasomal degradation. For example, SUMO-1 modified IκBα lysine 21 conferred the resistance to TNFα-induced ubiquitination and degradation of IκBα (190). However, it is now clear that SUMO-2/3 chains can act as signals for the recruitment of E3 ubiquitin ligase RING finger protein 4 (RNF4) that ubiquitylates SUMO chains and degrades the proteins modified with SUMO chains (191). For example, promyelocytic leukemia (PML) has been identified as the first substrate in the SUMO chain formation dependent ubiquitination and degradation pathway in response to arsenic (191-193). Further studies are needed to study PXR ubiquitination and SUMOylation crosstalk and determine the mutual effects between SUMO-2/3 chain and poly-ubiquitin chain formation.

Moreover, the relative reaction kinetics of SUMO-1 and SUMO-2/3 conjugation to PXR has not been studied. Based on current findings, we hypothesize that SUMO-2/3 conjugation is rapidly induced, whereas SUMO-1 modification acts later on. The rapid kinetics of SUMO-2/3 reaction may reveal highly dynamic regulation of the SUMO-2/3 modification in the context of inflammatory stress.

1.2 E3 Ligases

Initially not clear whether E3 ligases are required in the SUMOylation process because SUMO conjugation could take place in two enzymatic steps *in vitro*, including E1 and E2 (194). However, the majority of SUMOylation reactions in yeast are E3-dependent (162,195). Later, E3 ligases have been

shown to enhance SUMO attachment to many substrates both *in vitro* (163,196,197) and *in vivo* (164,198,199). Our *in vitro* SUMOylation assays revealed that PXR could be SUMOylated in two enzymatic steps *in vitro*, whereas PIASy enhanced the reaction capacity greatly.

We also suggest that both PIAS1 and PIASy could function as E3 ligases in cell-based assays. PIASy showed stronger E3 ligase functional activity on targeting both PXR and overall cellular substrates than that of PIAS1 *in vivo*. However, some previous studies indicated that although cells were over-expressed with the same amount of PIAS protein expression plasmids, the protein expression levels of each PIAS protein were different (200). Future work on analysis of PIAS1 and PIASy protein levels after transient transfection is required before we make the absolute conclusion that PIASy is a stronger E3 ligase to enhance PXR SUMOylation than PIAS1 *in vivo*.

The PIAS proteins have also shown preferences in selection of SUMO isoforms to be attached to PXR. We showed that PIAS1 and PIASy preferentially enhanced PXR modification by SUMO-3 rather than by SUMO-1. Another group has found that the transcription factor GATA-2 was SUMOylated and that PIASy could increase the extent of this modification, especially in the case of the SUMO-2 isoform (201). Therefore, it is tempting to speculate that PIAS proteins favor SUMO-2/3 over SUMO-1 in the SUMOylation reactions of transcription factors.

Besides the E3 ligating enzymatic activity, PIAS proteins have also been identified to regulate gene transcription through other mechanisms. PIASy has

been found to repress the transcriptional activity of AR independent of SUMOylation. It exerted repressive effects through the distinct repression domains and the recruitment of HDACs to the promoter region of AR target genes (202). In another case, PIAS1, but not other PIAS proteins, blocked the DNA binding activity of activated Stat1 and inhibited Stat1-mediated gene activation in response to interferon signaling (203). PIAS proteins-mediated subnuclear sequestration of transcription factors might represent another mechanism. For example, PIASy could target lymphoid enhancer factor 1 (LEF1) to nuclear bodies through the RING domain, which leads to the repression of LEF1 activity (163). It is unknown as to whether or not PIAS proteins could repress PXR trans-activation independent of SUMOylation through reducing PXR DNA binding activity, enhancing co-repressor recruitment, inhibiting the interaction with co-activator or subnuclear sequestration of PXR to nuclear bodies.

1.3 De-SUMOylation

In keeping with previous findings, our results support that the SENPs are functionally different in their ability to de-conjugate SUMOylated PXR. In particular, we found out that SENP3 and SENP5 prefer SUMO-3 monomeric conjugates. This is unlike SENP1 and SENP2, which can de-conjugate both SUMO-1 and SUMO-3. SENP2 removed SUMO conjugates completely; otherwise SENP6 efficiently removed SUMO-3 poly-chains.

The SENPs were shown to localize differently in cells. We need to further analyze the localization of SENPs in Hepa1-6 cells when cells are co-transfected

with PXR and SUMO plasmids. We postulate that in the absence of PXR, SENP1 shows primarily a nuclear localization. But SENP5 may reside mainly in nucleoli. By analyzing the co-localization of SUMOylated PXR and SENPs, we are able to distinguish the mechanism of their substrates specificity. If SENP1 was co-localized with SUMO-1-conjugated PXR and SENP5 was completely sequestered from SUMO-1-modified PXR, we might relate the different sub-cellular localization to the alternative isopepetidase activities of the SENPs.

Since SENPs are functionally different towards targets, the overall conjugation state of SUMO-1- and SUMO-2/3 modified proteins may in part be regulated at the level of de-conjugation rather than conjugation (204). The activity of SENPs was regulated by cellular conditions through control of their stability. For example, SENP3 was rapidly degraded under basal condition. In an oxidative state, the ubiquitin–proteasome pathway mediated degradation of SENP3 was inhibited. SENP3 re-distributed from the nucleolus to the nucleoplasm where it participated in the removal of SUMO-2/3 from a number of proteins under mild oxidative stress. Since SENP3 did not de-conjugate SUMO-1, the SUMO-1 conjugated protein level was not affected by oxidative stress stimulation (205). From this point of view, the different activity or stability of substrate-specific SENPs may account for the disparate SUMO conjugation patterns.

Previously, we found that the co-expression of SUMO-3 and Ubc9 in CV-1 cells had no effect on a PXR-response element luciferase reporter gene (XREM-LUC) (132). While SUMOylation of PXR did not inhibit its

trans-activation, little is known regarding the effect of de-SUMOylation of PXR by SENPs on transcriptional activation.

Several results revealed that de-SUMOylation by SENPs increased NRs transcriptional activity through different mechanisms. The studies discussed above (205) showed that the increased SENP3 activity under oxidative state de-SUMOylated p300, which is a co-activator of HIF1α. HIF1α is a master-regulator of stress-responsive genes (206) and SUMOylation of the p300 protein inhibited HIF1α transcription activity (207). Therefore, by down-regulating co-activator p300 SUMOylation level, SENP3 promoted HIF1α trans-activation of stress-responsive genes under oxidative stress (205). Another study on SENP1 and AR showed that SENP1 could increase AR transcriptional activation. It was not mediated by de-conjugation of SUMO from AR but through de-SUMOylation of HDAC1, a component of co-repressor complexes (208). HDAC1 is modified by SUMO-1 *in vivo*, which is essential for its repressive function (209). SENP1 inhibited the transcriptional repressive effect of HDAC1 through de-conjugating HDAC1 SUMO-1 modification, which in turn enhanced AR transcription activity (208). The effect of SENPs on PXR-mediated transcription activity has not been uncovered. Whether SENPs modulate PXR activity through de-conjugating SUMOylation of PXR itself or of its co-regulators is currently unknown. Therefore, it is of interest to study the potent regulatory activity of SENPs that modulate transcription systems in order to further understand the biological functions of SUMOylation/de-SUMOylation in a big picture.

1.4 PXR SUMOylation Sites

The bioinformatic approach to scan the sequence of PXR for the presence of a consensus SUMO acceptor site identified four potential sites within human PXR. Since the four sites are all located in the C-terminal hinge domain and LBD domain, we tested the SUMOylation capability of the recombinant protein composed of human PXR hinge and LBD parts *in vitro*. The hinge and LBD domains were SUMOylated by both SUMO-1 and SUMO-3 *in vitro* which is consistent with our hypothesis that SUMOylation sites reside in the C-terminal. However, it is likely that SUMO may be attached to non-consensus lysine residue, thus the total 28 lysine residues on PXR were analyzed systematically based on site-directed mutagenesis. The single, double, triple or quadruple PXR mutant was co-transfected with SUMO and PIAS plasmids in Hepa1-6 cells (data not shown). We revealed one potential site of human PXR SUMOylation at K108. By mutating K108 to arginine, the human PXR SUMO-1 and SUMO-3 conjugation levels were significantly down-regulated. After double mutation of K108 and K128, the SUMO-1 modification was completely lost, whereas the double mutant could still form monomeric conjugates by SUMO-3. The other SUMO-3 site(s) besides K108 and K128 has not yet been identified. It has been suggested that mutation both the lysine and an adjacent acidic amino acid might be required to inactivate some SUMO consensus sites (210,211). Further experimental design of site-directed mutagenesis based on this idea may help to solve the puzzle. According to our current data, the possible SUMOylation pattern on human PXR is summarized in the Table 1.

Table 1.

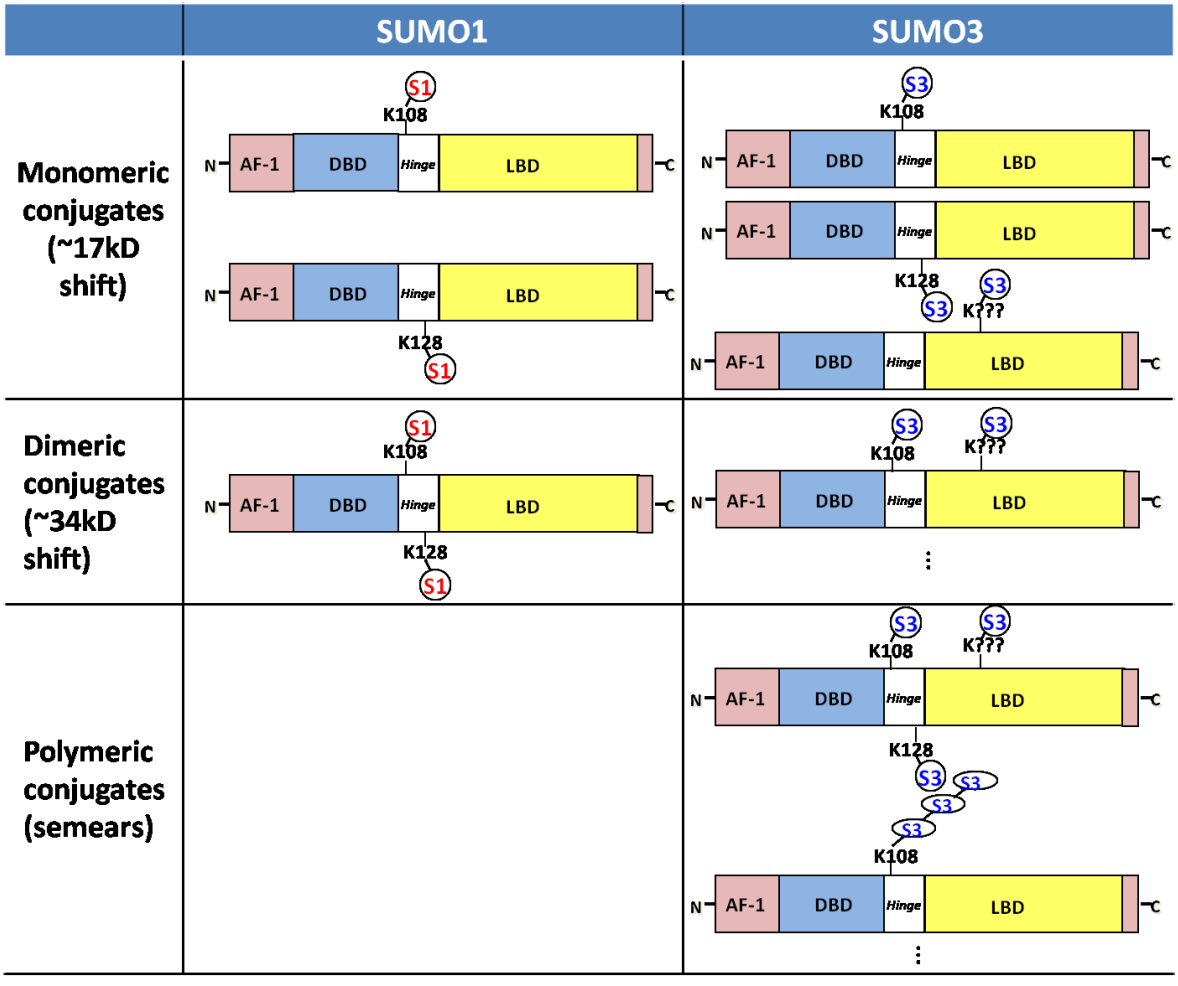


Table 1. PXR SUMOylation Pattern. The PXR protein is targeted by SUMO-1 at two major sites, K108 and K128. SUMO-1 modification leads to monomeric and dimeric conjugates. The SUMO-3 modification pattern is different from SUMO-1. There are at least three potential sites, including K108 and K128. The lysine residues could support mono-, multi-mono-, and poly-SUMO-3 conjugations of PXR.

The identification of PXR SUMOylation sites is of great value. It will facilitate our understanding of PXR SUMOylation biological functions and physiological roles. For example, it could provide a direct and strong basis to

test the hypothesis that PXR SUMOylation crosstalks with other post-translational modifications. It has been suggested that SUMO might compete with ubiquitin for the same lysine (190,212). Recent studies showed that PXR could be ubiquitinated for eventual proteasomal degradation (213,214). Future immunoblot analysis using the ubiquitin antibody could be employed to detect the capacity of wild type and SUMO(1)yaltion-deficient K108R K128R double mutant for ubiquitination. The mutant variant helps to determine if SUMO-1 conjugation antagonizes ubiquitin modification of PXR. Other interesting biological abilities could also be compared between PXR SUMOylation-deficient mutant and wild type form, including co-regulator interaction, heterodimerization with RXRα, sub-cellular localization, stability and regulation of target gene expression.

From a more clinical perspective, a knock-in mouse model harboring the un-SUMOylatable form of PXR could be generated to directly assess if PXR SUMOylation is essential for proper physiological function. Dr. Staudinger has generated a PXR-deficient mouse line in 2000 (43). Recent data from our lab showed that the PXR null mice exhibited signs of heightened inflammation in their small bowels with elevated NF-κB target gene expression (132). The knock-in mutant mice would directly test the extent to which SUMOylation regulates the *in vivo* anti-inflammatory function of PXR. Because the stoichiometry of PXR SUMOylation appears exceedingly low, the forced over-expression of SUMO-3 and PIASy we used in the studies could potentially lead to the production of experimental artifacts. Therefore, the PXR un-SUMOylatable mutant knock-in mice would allow us to overcome certain

drawbacks associated with using cell culture and are promisingly beneficial to directly test the physiological significances of PXR SUMOylation in liver and small intestine.

2. PXR Transrepression Model

Recent studies have revealed that ligand-activated transrepression of inflammatory response gene by PPARγ and LXRs is SUMOylation dependent (215,216). Our observations provide support to extend this transrepression pathway to an additional member of NR family: PXR. We showed that PXR transrepressed the induction of inflammatory response genes in Hepa1-6 cells in a ligand-independent manner. Since PXR was over-expressed in this case, it is questionable that over-expressed PXR represents the endogenous form of PXR in human liver cells which interacts with CCRP-HSP90 complex in the absence of ligand under basal condition. It is possible that PXR transrepression pathway is ligand-dependent in human hepatocytes because ligand activation is required for PXR dissociation from the CCRP-HSP90 complex and translocation to nucleus from cytoplasm. Taken together, our current hypothesized model is that activation of PXR by xenobiotic/endogenous compounds leads to the SUMOylation of a sub-population of this NR to stimulate the formation of transrepressive multi-protein complexes on the promoters of inflammatory response genes in liver and intestine.

However, there are some questions regarding the transrepression mechanisms that remain to be answered in the future. For example, it is likely that reduced target gene transcription is due to the down-regulated binding

capacity of transcription factor to its response element. So, is NF-κB DNA binding capacity altered in response to ligand-activated PXR SUMOylation? And what are the components of the co-repressor complexes formed on NF-κB? Which protein recruits and interacts with ligand-bound SUMOylated PXR? In order to further validate our model, some new experimental methods may be required to answer those questions.

Chromatin immunoprecipitation (ChIP) assay investigates the interaction between protein and DNA in the cells. Previously, ChIP assay has been used to detect the binding of the PXR-RXRα heterodimer to the regulatory sequences of *Cyp3a4*. The result showed that NF-κB activation disrupted the heterodimer DNA binding activity which led to suppression of PXR transcriptional activation (131). To further elucidate the mechanism underlying the suppression of pro-inflammatory gene expression by PXR activation, the ChIP assay could be performed to analyze NF-κB DNA binding activity before and after PXR activation which may account for the anti-inflammatory property of PXR.

The mammalian two hybrid assays allow us to determine the protein-protein interaction of human PXR with co-factors. According to our model, the ligand-activated PXR is SUMOylated and interacts with the co-repressor complexes on NF-κB to inhibit the co-repressors dissociation. However, during PXR trans-activation, the interaction between PXR and co-repressors is largely down-regulated after ligand binding. We are in the process of resolving this puzzle based on mammalian two hybrid system. We hypothesize that PXR interacts with different co-repressor proteins or different

domains of the same co-repressor in the trans-activation and trans-repression pathways.

The PXR transrepression model provides an explanation for how an agonist-bound PXR can be converted from an activator of gene transcription to a promoter-specific repressor of NF-κB target genes that regulate hepatic inflammation. Taken together, our results suggest that activation of PXR results in repression of inflammatory response gene, thus providing a mechanistic explanation for the long-term observed immunological suppression by rifampicin. We hypothesize that the inflammatory stimulation leads to PXR SUMOylation which stabilizes the co-repressor complexes on NF-κB, thereby causing transrepression of NF-κB signaling pathway.

3. Clinical Significance

Inflammatory bowel disease (IBD) is a relapsing and chronic inflammatory condition of the gastrointestinal tract occurring as ulcerative colitis (UC) or Crohn's disease (CD) (217). UC is limited to the colon, whereas CD can affect any part of the gastrointestinal tract with inflammation deeper into the layers of the intestinal wall (218). While the physiologic etiology of IBD is unclear, the prevailing view is that it is triggered by multiple factors, including the environment, genetic variations, intestinal microbiota, and disturbances in the innate and adaptive immune responses (219). Recent studies showed that PXR is associated with IBD and decreased expressions of PXR target genes cause loss of detoxification and removal of xenobiotics in the gut (220). The immunosuppressive and anti-inflammatory role of PXR activation provides a

new drug target for treating IBD. Rifaximin, a gut-specific human PXR activator, has been shown to be beneficial in the treatment of UC and moderate CD (221-223). A recent study on clinical use of rifaximin suggests that rifaximin down-regulates NF-κB target gene expression in the PXR-dependent manner and protects against IBD in the patients (224). It is likely that the transrepression effect of NF-κB signaling in IBD might be mediated by ligand-dependent SUMOylation of human PXR. And our study on PXR SUMOylation provides a molecular basis for the rifaximin treatment of IBD in the gut.

However, the activation of PXR could lead to transactivation of PXR target genes that are involved in multiple physiological processes. The changes in the DMEs and drug transporter proteins would cause various adverse responses. One major side-effect of PXR activators is the ADRs. There is a clear recognition that the frequency of ADRs is increased in patients affected with an underlying systemic inflammation. The long-term treatment is often limited by adverse side effects believed to be caused by PXR-mediated gene transcription. This has led to the pursuit of compounds that retain the anti-inflammatory properties yet lack the adverse side effects associated with traditional PXR activators. New molecular insights regarding the biology of PXR SUMOylation will provide new opportunities to develop novel pharmacological strategies for addressing ADRs, and will eventually help to identify small molecules that will be used to treat inflammatory liver diseases.

4. Conclusion

In summary, the potential for PXR SUMOylation involvement in inflammatory response is far-reaching and requires additional investigation to elucidate the specific transrepression mechanism. Regardless, PXR SUMO modification and/or de-SUMOylation are involved in the crosstalk with NF-κB signaling pathway to exert its physiological functions. These studies provide molecular basis and the biochemical details of how PXR is converted from a positive regulator of drug metabolism into a transcriptional suppressor of inflammation in liver tissue. Novel molecules that specifically promote entry of PXR into the SUMOylation-dependent transrepression pathway may be of great therapeutic utility in liver diseases in which inflammation plays a significant pathogenic role.

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