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Post-translational Modification of Pregnane X Receptor

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Abstract

Pregnane x receptor (PXR, NR1I2) was originally characterized as a broad spectrum entero-hepatic xenobiotic ‘sensor’ and master-regulator of drug inducible gene expression. A compelling description of ligand-mediated gene activation has been unveiled in the last decade that firmly establishes this receptor’s central role in the metabolism and transport of xenobiotics in mammals. Interestingly, pharmacotherapy with potent PXR ligands produces several profound side effects including decreased capacities for gluconeogenesis, lipid metabolism, and inflammation; likely due to PXR-mediated repression of gene expression programs underlying these pivotal physiological functions. An integrated model is emerging that reveals a sophisticated interplay between ligand binding and the ubiquitylation, phosphorylation, SUMOylation, and acetylation status of this important nuclear receptor protein. These discoveries point to a key role for the post-translational modification of PXR in the selective suppression of gene expression, and open the door to the study of completely new modes of regulation of the biological activity of PXR.

Keywords

Pregnane x receptor; Post-translational modification

Introduction

Pregnane x receptor (PXR, NR1I2) was originally identified on the basis of its sequence homology with other nuclear receptor (NR) proteins in the expressed sequence tag database. The full-length mouse PXR cDNA was characterized in 1998 using the expressed sequence tag to screen a mouse liver cDNA library, and the receptor was named PXR based on its activation by pregnane (21-carbon) steroids [1]. Shortly after its discovery, PXR was classified as a broad specificity receptor that is activated by a wide variety of drugs and xenobiotic compounds as a heterodimer with RXR α . Upon ligand binding the PXR-RXR heterodimer binds to multiple sites on the cytochrome P450 3A (*CYP3A*) promoter and activates gene expression and provides the molecular basis for the induction of *CYP3A* gene expression by xenobiotics [1–3].

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PXR Ligands

Numerous ligands for PXR have been identified across various species, and it is now well accepted that a species-specific PXR-activation profile exists. For example, mouse and rat PXR are activated by the *CYP3A* inducer pregnenolone 16 α -carbonitrile (PCN), whereas PCN has little effect on human and rabbit PXR. On the other hand, rifampicin (Rif) activates human and rabbit PXR but has virtually no effect on the mouse and rat receptors [3–6]. In fact, PXR is activated by a broad range of lipophilic compounds including a myriad of synthetic and endogenous steroids, certain bile acids, and a variety of drugs and plant products. In contrast to the classic steroid hormone receptors, high-affinity (sub-nanomolar) ligands for PXR have not been discovered. For example, the lowest EC₅₀ values of steroids that activate PXR are low-micromolar, generally two to three orders of magnitude higher than concentrations found in circulating plasma [5, 6].

PXR-target Genes

PXR ligands have been shown to stimulate expression of genes that encode enzymes involved in the oxidation (phase I), conjugation (phase II) and transport (phase III) of xenobiotics. The first genes shown to be directly regulated by ligand-mediated PXR activation were *CYP3A* family members in both mouse and human liver and intestine [1, 3]. Additional phase I drug metabolism gene products regulated by PXR include numerous cytochrome P450s, aldehyde dehydrogenases, alcohol dehydrogenases, carboxylesterases, and several enzymes involved in heme production and support of the CYP cycle such as aminolevulinic acid synthase and P450 oxidoreductase [7, 8]. Phase II drug metabolism gene products regulated by PXR activation include UDP-glucuronosyl-transferases, sulfotransferases and glutathione S-transferases [8–13]. Finally, phase III drug transporters gene products regulated by PXR include numerous ATP-binding cassette membrane pumps of the multidrug resistant family and organic anion transporting protein 1A4 in rodents [14–16].

Negative Physiological Functions of PXR

While the molecular basis for ligand-mediated PXR gene activation programs controlling drug metabolism and drug transport activity is relatively well described, much less is known about the molecular mechanisms governing the observed ligand-dependent repressor function of the PXR protein. Recent research efforts indicate that ligand-mediated activation of PXR negatively regulates several key biochemical functions in liver and intestine including the synthesis of glucose, ketone bodies, β -oxidation and transport of lipids, as well as inflammatory processes (Figure 1). The general mechanism for drug-mediated repression of these important physiological functions appears to involve protein-protein interactions between liganded-PXR and the transcription factors and accessory proteins required for driving full-activation of respective programs of gene expression. The molecular basis for reciprocity between these biochemical pathways is currently the focus of several research groups, and the biochemical details are currently emerging.

Glucose Homeostasis

Glucose production by liver is tightly controlled by the insulin and glucagon signaling pathways. These counter-regulatory signaling pathways play a critical role in survival during fasting and starvation by regulating the transcription of key target genes comprising the gluconeogenic gene expression program including glucose-6-phosphatase (*G6Pase*) and phosphoenolpyruvate carboxykinase (*PEPCK*). Glucagon increases glucose production by up-regulating the transcription of key genes that encode the rate-limiting enzymes in the gluconeogenic pathway, whereas insulin signaling rapidly suppresses the expression of the

genes encoding these tightly regulated enzymes. The CREB protein is a cellular transcription factor that binds to certain DNA sequences called cyclic AMP response elements (CREs), thereby increasing the transcription of downstream genes. Glucagon stimulates cyclic AMP-dependent protein kinase (PKA) that phosphorylates CREB. PKA-phosphorylated CREB binds to CREs and activates the transcription of genes that contain CREs in their promoter such as *G6Pase* and *PEPCK1*. Phosphorylated CREB then transactivates the expression of *G6Pase* and *PEPCK*.

Previous observations have revealed functional links between glucose metabolism and PXR-mediated signaling pathways. For example, it is known that PXR ligands repress expression of *G6Pase* and *PEPCK* [17–19]. Treatment with the potent rodent PXR activator PCN decreased blood glucose levels in fasting wild-type mice, but not in PXR-null mice [19]. Moreover, the genes that encode *G6Pase* and *PEPCK* are decreased in transgenic mice that express a constitutively activated form of human PXR [20]. These data suggest that sustained PXR activation actively represses the gluconeogenic pathway through interference with or sequestration of transcription factors and protein cofactors that are involved in transcriptional regulation.

Forkhead box protein O1 (FOXO1) belongs to the forkhead family of transcription factors which are characterized by a distinct fork head domain. In hepatic cells, a dephosphorylated form of FOXO1 drives the transcription of *G6Pase* and *PEPCK* and its presence in the nucleus is required for full activation of the gluconeogenic program of gene expression. Insulin signaling activates the phosphatidylinositol 3-kinase-Akt pathway to phosphorylate FOXO1, excluding it from the nucleus and resulting in the insulin-dependent repression of *G6Pase* and *PEPCK* [21, 22]. Interestingly, FOXO1 has been shown to interact with several NR proteins to function as either a transcriptional corepressor or coactivator protein [23, 24]. Moreover, the FOXO1 protein was found to function as a coactivator of PXR-mediated gene activation. In contrast, ligand-mediated activation of PXR suppressed FOXO1 transcriptional activity by preventing binding to its response element in target genes such as *G6Pase* and *PEPCK* [25].

It has also been proposed that PXR inhibits the expression of gluconeogenic enzymes by interfering with CREB signaling. PXR activation results in the repression of CREB-mediated activation of the *G6Pase* promoter in both mice and in a human hepatocarcinoma cell line. This apparently occurs through the binding of liganded-PXR protein directly to CREB, which thereby prevents CREB interaction with the CRE on the *G6Pase* promoter [19].

The NR coactivator protein peroxisome proliferator-activated receptor gamma coactivator 1-alpha (PGC-1 α) is induced by glucagon and coactivates hepatocytes nuclear factor 4 α (HNF-4 α)-mediated transcription of *G6Pase* and *PEPCK*. Ligand-activated PXR dissociates PGC-1 α from HNF-4 α through a direct competition/squelching mechanism, thereby repressing the transcription of *PEPCK* and *G6Pase* [18]. Since PGC-1 α is also a co-factor for CREB- and FOXO1-mediated expression of gluconeogenic-target genes, a similar mechanism implicating sequestration of PGC-1 α from these two transcription factors by PXR is likely to be responsible for drug-mediated repression of gluconeogenesis. Hence, the underlying molecular mechanism of PXR-mediated repression of glucose production appears to be the direct binding of PXR to transcription factors and accessory proteins that activate gene expression programs critical for the gluconeogenesis such as FOXO1, CREB, HNF4 α , and PGC-1 α .

Lipid Metabolism and Ketogenesis

It is well known that treatment with drugs, now classified as PXR activators, affect lipid metabolism in patients. For example, treatment with Rif or carbamazepine can induce hepatic steatosis, characterized by the abnormal accumulation of triglycerides in liver [26, 27]. It appears that drug- and lipid-metabolism are interconnected through a complex network of transcriptional regulators that include PXR. The role for PXR in the development of hepatic steatosis raises some concern regarding the development and safety of drugs that are potent PXR ligands. Overall, the role of PXR in lipid metabolism and steatosis warrants further investigation, however, recent studies indicate a clear role for this receptor in the regulation of hepatic lipid metabolism.

When blood glucose is low, the liver metabolizes fatty acids via β -oxidation to provide ketone bodies to extra-hepatic tissues. Forkhead box A2 (FOXA2) has been shown to positively regulate this process by controlling the transcription of target genes including carnitine palmitoyltransferase 1A and 3-hydroxy-3-methylglutaryl-CoA synthase 2 [28, 29]. It has been suggested that ligand-activated PXR represses hepatic energy metabolism by decreasing both β -oxidation and ketogenesis. Treatment with PCN down-regulates the expression of genes encoding carnitine palmitoyltransferase 1A and 3-hydroxy-3-methylglutaryl-CoA synthase 2 in wild type, but not in PXR-null mice. It was further shown that activated PXR and FOXA2 physically interact through their ligand- and DNA-binding domains, respectively. This interaction prevents FOXA2 from binding to its response elements and leads to the repression of carnitine palmitoyltransferase 1A and 3-hydroxy-3-methylglutaryl-CoA synthase 2 [30]. In addition, it has been shown that HNF-4 α directly regulates expression of carnitine palmitoyltransferase 1A [31]. It has been demonstrated that PXR interferes with HNF-4 α signaling by targeting PGC-1 α and producing a squelching effect [18]. Since HNF-4 α and PGC-1 α are jointly involved in the regulation of carnitine palmitoyltransferase 1A it is likely that crosstalk with ligand-activated sequestering by PXR applies to this gene promoter as well through its interaction with FOXA2.

Inflammatory Response

Exposure to xenobiotics can impair immune function. In fact, it is a long-standing observation that Rif tends to suppress immunological responses in liver cells [32–34]. Recent publications have demonstrated a mutual inhibition between PXR and the inflammatory mediator nuclear transcription factor kappa B (NF- κ B), thus providing a potential molecular mechanism that links xenobiotic metabolism and inflammation [35, 36]. Activation of PXR by Rif suppresses the expression of typical NF- κ B target-genes such as cyclooxygenase-2, tumor necrosis factor α (*TNF α*), intercellular adhesion molecule-1 and several interleukins [36]. Conversely, NF- κ B activation by lipopolysaccharide and TNF α results in the suppression of CYP3A activity through interactions of NF- κ B with the PXR-retinoid-x-receptor complex [37]. Furthermore, hepatocytes derived from PXR-null mice have elevated NF- κ B target-gene expression compared to hepatocytes from wild-type mice. The PXR-null mice also exhibit heightened signs of inflammation in their liver and small bowel [36, 38]. This could be due to the loss of negative regulation of NF- κ B activity following PXR activation, or is perhaps due to inadequate clearance of toxic substances in the absence of PXR.

Several fundamental questions remain regarding the molecular mechanisms of PXR-mediated gene repression. For instance, (1) does the selective interaction of liganded-PXR with transcription factors and accessory proteins involve post-translational modification of the PXR protein? (2) Is the selective repression of specific programs of gene expression dependent upon modification-mediated conformational change of the PXR protein? (3) How do ubiquitination, phosphorylation, SUMOylation, and acetylation of PXR integrate to affect

PXR-target gene activation and subsequent biochemical functions in the entero-hepatic system? It is therefore important to briefly review the direct evidence for post-translational modification of PXR, and also discuss the likely interplay of ligand binding with the ubiquitylation, phosphorylation, SUMOylation, and acetylation status of PXR.

Ubiquitination of PXR

While degradation is known to play an important role in NR function [39], relatively little is known about the degradation of PXR. PXR was found to interact with suppressor for gal-1, a key component of the 26S proteasome complex, in the presence of progesterone but not in the presence of endocrine disrupting chemicals [40]. A follow up study confirmed that PXR is differentially degraded in response to progesterone when compared with endocrine disrupting chemicals [41]. This finding suggests that proteasomal-mediated PXR degradation may be differentially affected by various PXR agonists. The extent to which ubiquitination and/or degradation of PXR protein affects glucose, lipid, ketone body, and inflammatory status in mammals is worthy of further investigation.

Our laboratory has recently developed a cell-based over-expression and western blot experimental approach for direct detection of ubiquitinated PXR (Figure 2A). As expected, detection of ubiquitinated PXR protein is dramatically increased in response to pharmacological inhibition of 26S proteasome activity with MG132 (Figure 2B, *lane 4*). Interestingly, forced activation of the PKA signaling pathway selectively increases the ubiquitination of PXR (Figure 2B, *lane 6, note the asterisks*). Notably, pharmacological inhibition of the proteasomal degradation pathway abolishes PXR transactivation of the CYP3A4 promoter in reporter gene transfected CV-1 cells (Figure 2C). This is consistent with an ubiquitin-dependent promoter clearance mechanism, and is highly reminiscent of recent reports detailing similar modes of regulation of NR proteins peroxisome proliferator-activated receptor gamma (PPAR γ , NR1C3) and liver-x receptor alpha/beta (LXR α/β , NR1H3/NR1H2) [42–44]. The interaction between PXR and the ubiquitin signaling pathway appears to be relatively complex and warrants further investigation.

Phosphorylation of PXR

Protein phosphorylation plays an important role in the regulation of PXR function [45]. Treatment of mouse hepatocytes with the cyclic AMP-dependent protein kinase (PKA) activator 8-Bromo-cyclic AMP increased the induction of *Cyp3a11* by the PXR agonist taxol and enhanced the binding of mouse PXR to the transcriptional coactivator proteins Steroid Receptor Coactivator-1 and Peroxisome Proliferator-activated Receptor (PPAR)-binding Protein. Furthermore, kinase assays show that PXR can serve as a substrate for catalytically active PKA *in vitro*, suggesting one potential mechanism for PKA-mediated modulation of *CYP3A* gene expression [46]. Additionally, there appears to be significant species differences in the effect of kinase signaling pathways. For example, while PKA activation increases PXR activity in mouse hepatocytes, it serves as a repressive signal in both human and rat hepatocytes. Similar to the PXR-ligand response, this suggests a species-specific effect for the modulation of drug-inducible *CYP3A* gene expression by PKA signaling [47].

Activation of protein kinase C signaling by phorbol myristate acetate repressed PXR activity in reporter gene assays and in hepatocytes by increasing the strength of interaction between PXR and the nuclear receptor corepressor (NCoR) protein, and by abolishing the ligand-dependent interaction between PXR and Steroid Receptor Coactivator-1. Interestingly, the protein phosphatase PP1/2A inhibitor okadaic acid strongly represses PXR-dependent transactivation [48]. In addition, cyclin-dependent kinase 2 (Cdk2) attenuated the activation of *CYP3A4* gene expression. PXR is a suitable substrate for the Cdk2 enzyme *in vitro*, and a

phosphomimetic mutation at a putative Cdk2 phosphorylation site at (S350D) impaired the function of human PXR, whereas a phosphorylation-deficient mutation (S350A) conferred resistance to the repressive effects of Cdk2 on a reporter gene in HepG2 cells [49]. The results of these studies confirm that the activity of PXR is modulated by changes in its overall phosphorylation status. Determining whether phosphorylation of PXR at specific sites influences the integration between cell-signaling pathways and PXR-mediated repression remains an open and important question for future research.

SUMOylation of PXR

Long-term treatment of patients with Rif inhibits the inflammatory-response in liver [34, 37]. Though the molecular basis for this phenomenon has remained obscure, it was recently predicted that it should involve SUMOylation of PXR in intestine and liver [50]. We have demonstrated that activation of the inflammatory response in hepatocytes strongly modulates the SUMOylation status of ligand-bound PXR [38]. The SUMOylated PXR protein contains SUMO2/3 chains and feedback represses the immune response in hepatocytes and likely in intestinal tissue as well. Future studies of SUMOylation are expected to provide a novel paradigm that uniquely defines the molecular basis of the interface between PXR-mediated gene activation, drug metabolism and inflammation in intestine and liver tissue.

A non-biased approach for identification of the sites and molecular mechanisms of PXR SUMOylation is badly needed. We have therefore designed a strategy that is based upon a very recent report in the literature [51]. Our experimental approach utilizes a forced over-expression cell-based assay and is depicted in figure 3A. A novel SUMO expression construct based on the amino acid sequence of SUMO-3 encodes a protein which we have termed SUMO-X, and the amino acid substitutions are depicted in figure 3B.

The SUMO-X protein incorporates several key features to allow non-biased enrichment and identification of SUMOylated PXR peptides produced *in vitro*, in cultured cells, or in live animals. This novel strategy creates an identifiable diglycyl lysine signature tag on SUMOylated PXR peptides that will be detected by mass spectrometry. The key amino acid substitutions in SUMO-X are depicted in red lettering in Figure 3C. The SUMO-X contains an N-terminal 6X-histidine-tag that allows enrichment of total SUMOylated substrates from an *in vitro* mixture or from whole-cell lysate (Figure 3C, **Step 1**). Cleavage of SUMOylated proteins with the LysC protease will produce predictable branched peptides as a result of the substitution mutation in SUMO-X at position 82 (T82K) in combination with the lysine residues contained in SUMO-substrates. The substitution of valine and glutamine amino acid residues in SUMO-3 with cysteine residues at positions 85 and 87 in SUMO-X (V85C and Q87C) creates a unique second affinity-tag for use with thiopropyl sepharose beads (Figure 3C, **Step 2**). This second enrichment step will allow immobilization of the desired SUMOylated peptides from a complex mixture. The site-directed mutation in SUMO-X at position 90 (T90K) produces a unique Trypsin cleavage site and leaves the diglycyl lysine tag intact on the SUMOylated peptides. The resulting modified peptides will then be detected using a mass spectrometry-based approach. The SUMO-X reagent is adaptable to expression using viral vectors for subsequent transduction of primary cultures of hepatocytes, as well as for mouse model systems using tail vein injection methods for delivery and isolation of the SUMO-X-labeled substrate proteins *in vivo*.

This novel experimental approach should overcome the observed low stoichiometry of SUMOylated substrates within cells, and will likely provide a non-biased molecular tool for identification of novel signal-mediated SUMO-3 substrates. When coupled with a proteomic approach, this 2-step enrichment strategy has previously been used to identify substrates and non-consensus SUMO-1 sites in cells [52]. As a proof-of-concept, we show here that

Ubc9 increases SUMOylation of PXR by SUMO-3 in transfected HeLa cells. Interestingly, over-expression of E3-SUMO-ligase family members PIAS1 and PIAS4 also increases SUMOylated form of PXR in cultured HeLa cells (Figure 3D, *compare left panel and middle panel*).

When SUMO-X is substituted for SUMO-3, we observe a similar result (Figure 3D, *right panel*). A close examination of the SUMO-modified form of PXR reveals that PIAS4 functions as a more effective E3 ligase enzyme when compared with PIAS1. Moreover, Ubc9, PIAS1, and PIAS4 can increase SUMO-modified forms of PXR independently. Finally, we note that SUMO-X does not appear to support chain formation on PXR as efficiently as wild-type SUMO-3, with the SUMO-X construct supporting mainly two primary sites of modification (Figure 3, *note asterisks*). This is highly reminiscent of NR PPAR γ that also has two primary sites for SUMOylation (K77 and K365). It is interesting to note that only one of the SUMOylation sites (K365) serves as the functional link between ligand-activated PPAR γ and its ability to transrepress NF- κ B activity [43].

Acetylation of PXR

Recent evidence suggests that down-regulation of P300-induced farnesoid-x-receptor (FXR, NR1H4) acetylation alters expression of FXR-target genes involved in lipoprotein and glucose metabolism [53]. A more recent follow-up study indicates that FXR is a target of silent mating type information regulation 2 homolog-SIRT1, a protein deacetylase that mediates nutritional and hormonal modulation of hepatic energy metabolism. The lysine residue in FXR at position 217 (K217) is the major acetylation site targeted by p300 and SIRT1. Acetylation of FXR increases its stability but inhibits heterodimerization with RXR α , DNA binding, and transactivation activity [54]. By analogy, PXR is also the likely target of acetylation, however, the extent to which PXR is targeted by SIRT1 or p300 is currently unknown. An experimental approach using 6X-histidine-tagged human PXR coupled with western-blotting analysis utilizing antibodies that recognize acetyl-lysine has been recently developed (Figure 4A). Acetylation of PXR is readily detected using this experimental approach (Figure 4B). These recently published data strongly suggests that PXR is acetylated at some level [55]. The effect of PXR acetylation and metabolic status on ligand-mediated PXR gene activation pathways is currently not well characterized. Interestingly, decreased capacity for drug metabolism is observed in patients with morbid obesity, hepatic steatosis, and non-alcoholic steatohepatitis [56–58]. Future research efforts should seek to determine the extent to which acetylation of PXR is involved in crosstalk between drug metabolism and energy metabolism.

Conclusions

PXR was originally characterized as a regulator of the homeostatic control of steroids, bile acid, and xenobiotics. However recent evidence has revealed a negative regulatory role for PXR in gluconeogenesis, lipid metabolism, and inflammation through either direct regulation or signal-activated crosstalk with other transcription factors. Ligand binding is the primary mode of PXR activation, but several signaling pathways also interface with PXR and affect its overall responsiveness to environmental stimuli, likely by altering the post-translational modification status of PXR and subsequent interaction with its associated protein partners. The extent to which competitive post-translational modifications of PXR at individual lysine residues by SUMO-, Acetyl-, and ubiquitin-modification is strongly suspected; however, the data are just beginning to emerge.

Crosstalk between phosphorylation, SUMOylation, ubiquitination or acetylation has been demonstrated in studies of other transcriptional regulators including NF κ B and p53 [59, 60], and this area needs to be further explored with respect to post-translational modification of

PXR. Moreover, virtually nothing is known about the signal- or cell-type-dependent regulation of the machinery involved in generating these post-translational modifications with respect to PXR. It is well known that various cellular stresses including heat shock, osmotic stress, and reactive oxygen species can globally affect SUMO conjugation and deconjugation when examined using a proteomic approach [61–63]. Whether specific changes in post-translational modification of PXR also occurs in response to metabolic, pathogenic, and xenobiotic stress associated with diseases and infection remains an interesting future issue to be explored. Finally, pharmacological manipulation of the complex network of factors that contribute to PXR activity present therapeutic opportunities in the treatment of numerous diseases including lipid and inflammatory disorders.

Abbreviations

PXR	Pregnane x receptor
NR	nuclear receptor
PCN	pregnenalone 16 α -carbonitrile
Rif	rifampicin
G6Pase	glucose-6-phosphatase
PEPCK	phosphoenolpyruvate carboxykinase
CYP3A	cytochrome P450 3A
CREs	cyclic AMP response elements
PKA	cyclic AMP-dependent protein kinase
PGC-1α	peroxisome proliferator-activated receptor gamma coactivator 1-alpha
HNF-4α	hepatocytes nuclear factor 4 α
NF-κB	nuclear transcription factor kappa B
TNFα	tumor necrosis factor α
Cdk2	cyclin-dependent kinase 2
FXR	farnesoid-x-receptor
SIRT1	silent mating type information regulation 2 homolog-1
PPAR	peroxisome proliferator antigen receptor
LXR	liver x receptor
FOXO1	Forkhead box protein O1
FOXA2	Forkhead box A2

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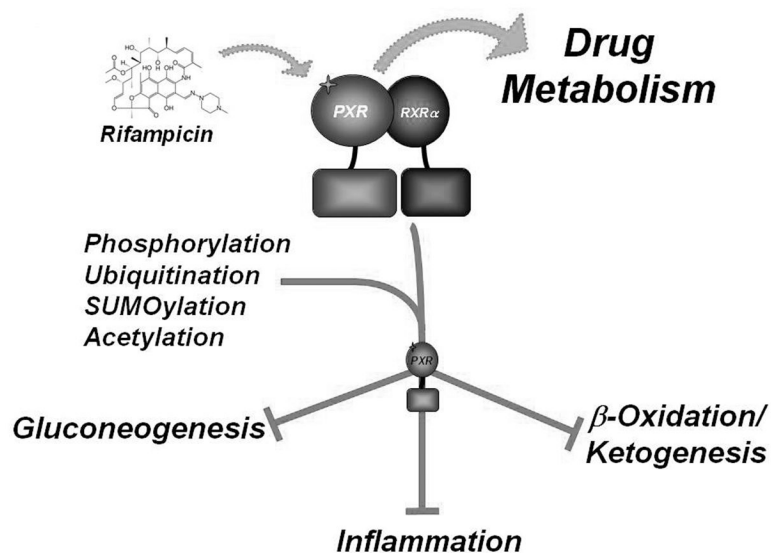


Figure 1. Negative Regulatory Roles of PXR

PXR was originally characterized for its role in xenobiotic and endobiotic detoxification. However, recent evidence has described a role for PXR in glucose and lipid homeostasis, as well as in repression of inflammatory programs of gene expression. A central role for post-translational modification of PXR is hypothesized to selectively repress biochemical pathways in liver and intestine.

Figure 2A

6X-His-Ub Pull-down

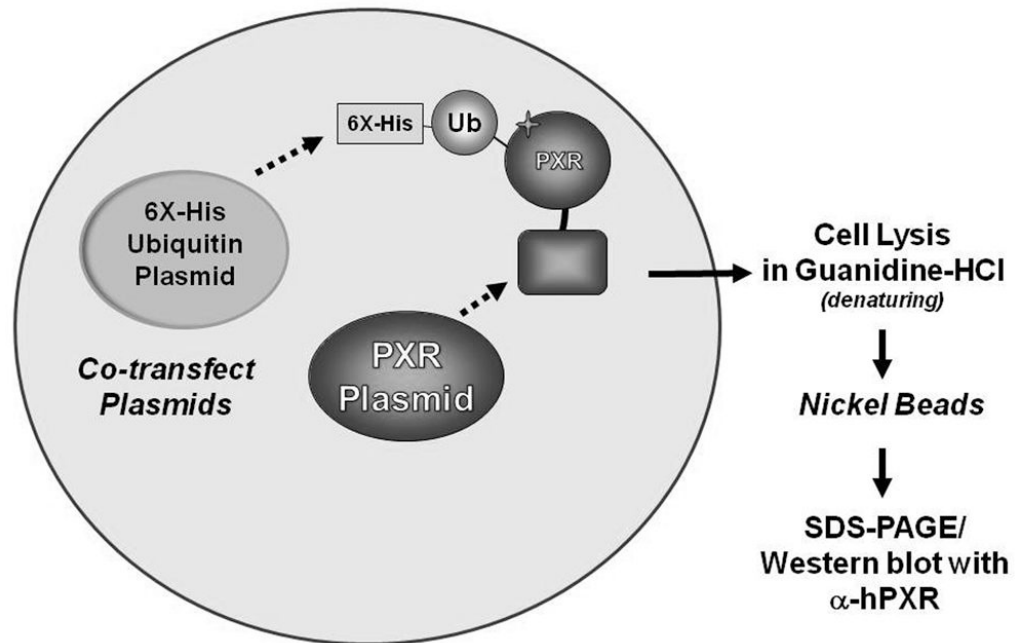


Figure 2B

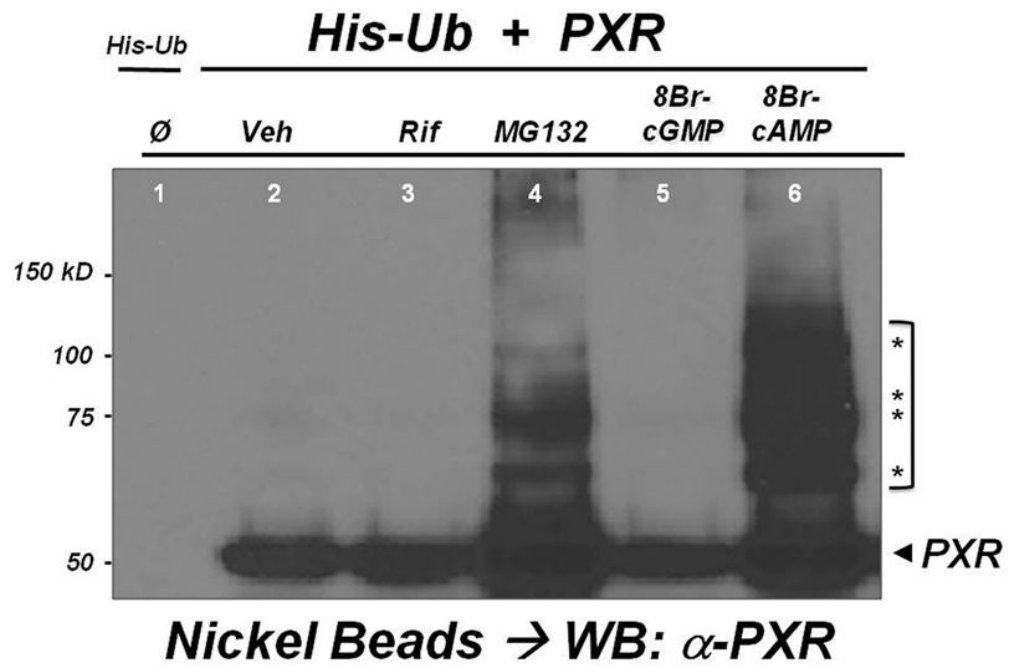
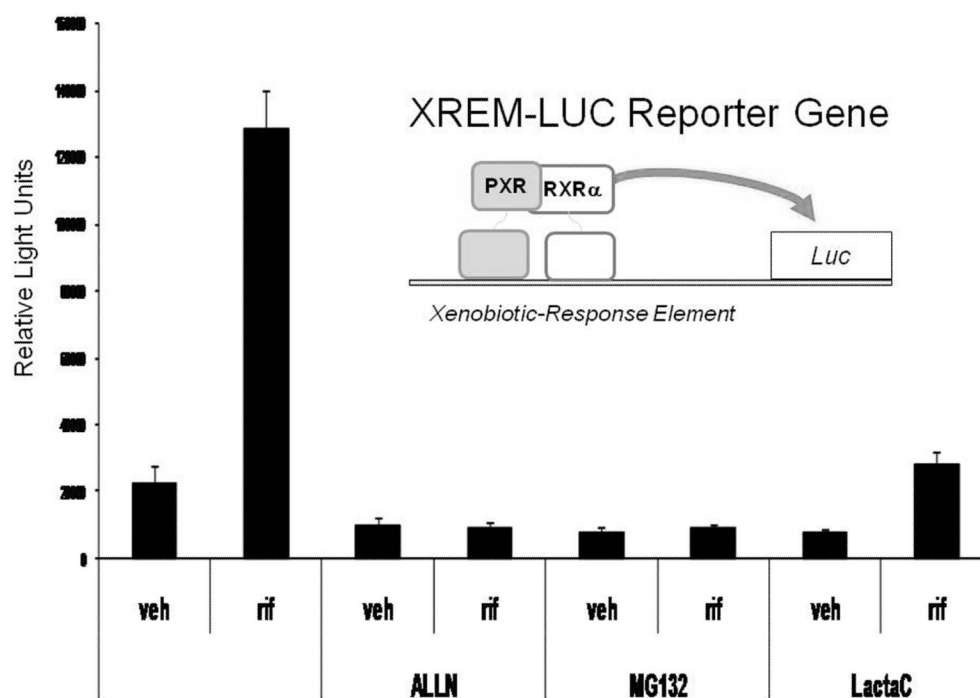


Figure 2C

**Figure 2. Ubiquitination of PXR**

(A) Mammalian expression vectors encoding affinity-tagged (6X-histidine) ubiquitin and human PXR are introduced into cultured HeLa cells using lipofectamine as described [36]. Twenty-four hr post-transfection, cells were treated with Rif (10 μ M), MG132 (25 μ M), 8-Bromo cyclic AMP (0.5 mM, 8-Br- cAMP), or 8-bromo cyclicGMP (0.5 mM, 8-Br- cGMP) for an additional 24 hr. Whole-cell lysates were generated using denaturing conditions as described [64]. (B) Western blotting was performed using a monoclonal antibody specific for human PXR (sc-48340, Santa Cruz). (C) The PXR-dependent XREM-LUC reporter gene [2] was transfected together with an expression vector encoding human PXR. Twenty-four hr post-transfection, cells were treated with vehicle, Rif (10mM), ALLN (100 μ M), MG132 (25 μ M), or lactacystin (10 μ M) for an additional 24 hr. The data are reported as relative light units and represent the mean of assays performed in triplicate \pm SE and is normalized to β -galactosidase activity.

Figure 3A

6X-His-SUMO Pull-down

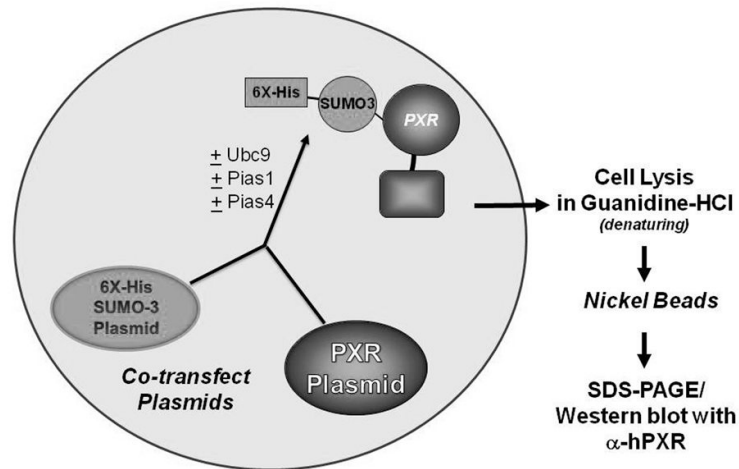


Figure 3B

SUMO-X is an An Engineered Form of SUMO-3

SUMO-X MSEEKPKEGVKTENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYSERQGLSM 54
 SUMO-3 MSEEKPKEGVKTENDHINLKVAGQDGSVVQFKIKRHTPLSKLMKAYCERQGLSM 54

SUMO-X RQIRFRFDGQPINETKTPAQLMEDEDEKIDCFQQRGG 92
 SUMO-3 RQIRFRFDGQPINETDTPAQLMEDEDEDTIDVFQQQTGG 92

C47S, D70K, T82K, V85C, Q87C, T90R

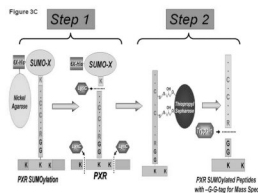
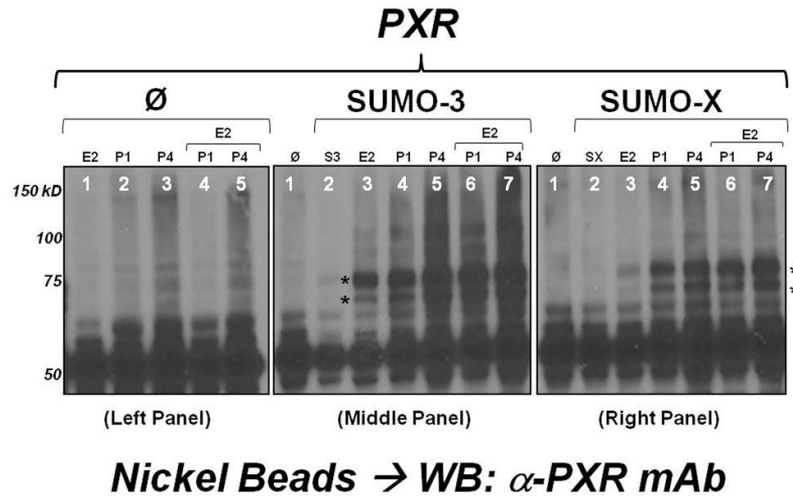


Figure 3D

**Figure 3. SUMOylation of PXR**

(A) Mammalian expression vectors encoding affinity-tagged (6X-histidine) SUMO-3 and human PXR are introduced into cultured HeLa cells in the presence and absence of E2 ligase (Ubc9), and the E3 ligase enzymes PIAS1, or PIAS4. Forty-eight hr post-transfection, whole-cell lysates were generated as described [64]. (B) The SUMO-X protein incorporates several key point mutations to allow non-biased enrichment and identification of SUMOylated PXR peptides when expressed together with substrates in vitro, in cultured cells, or in live animals. (C) Key altered amino acid residues are colored in **RED**. Sumo-targeted Lysine residues are protected from digestion with LysC protease. The SUMOylated peptide of interest is indicated with **GOLD** color. **STEP 1.** The PXR protein is SUMOylated and enriched using Nickel-agarose beads. After washing, LysC cleavage results in the release of the Cysteine tag from the rest of SUMOX. The substrate is also digested, but the SUMOylated Lysine residue is protected from cleavage. **STEP 2.** Using Thiopropyl sepharose, the SUMOylated cysteine peptides are covalently retained. The target peptides are eluted with trypsin digestion, and the diglycine (GG)-modified target lysine in PXR is identified as a SUMOylation site using LC-MS/MS. (D) The cDNAs encoding His-SUMO-3, His-SUMOX, Ubc9, PIAS1, or PIAS4 were transfected alone or together as indicated. Isolated PXR protein was detected using a monoclonal antibody that recognize human PXR.

Figure 4A

6X-His-PXR Pull-down

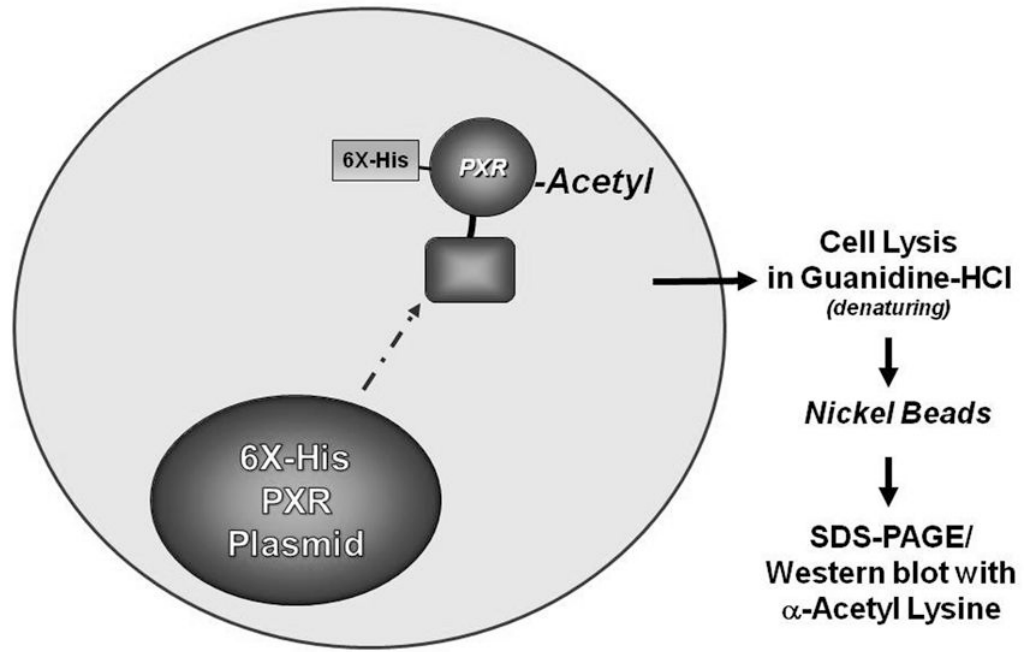


Figure 4B

293T

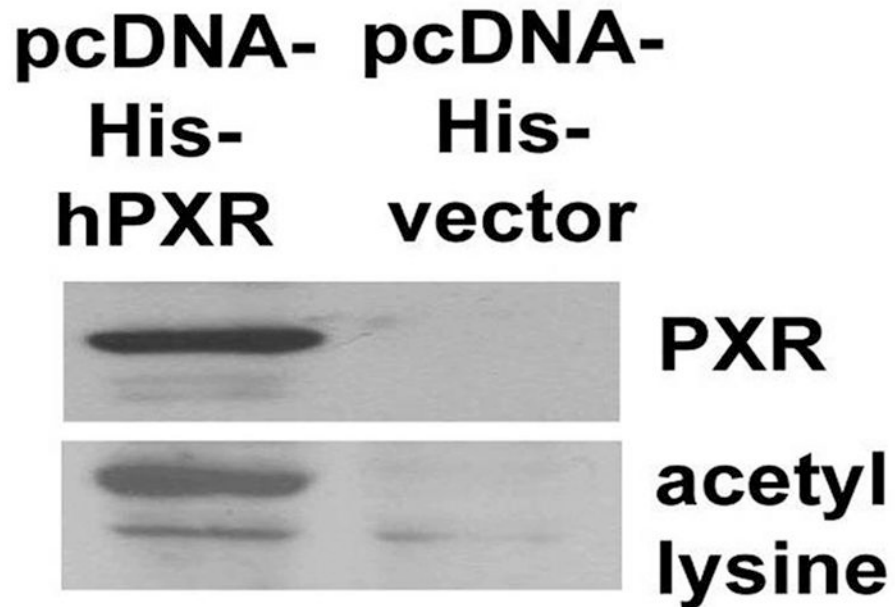


Figure 4. Acetylation of PXR

(**A**) Cultured 293T cells are transfected with either a His-tagged human PXR expression construct (pCDNA-His-hPXR) or an empty pCDNA-His vector plasmid as a negative control. (**B**) Captured proteins from nuclear extracts were subjected to SDS-PAGE and subsequent western blot analysis using an antibody that recognizes either human PXR (*Top Panel*) or acetyl-lysine (*Bottom Panel*).