**REGULATION OF DRUG METABOLISM AND INFLAMMATION BY PREGNANE X RECEPTOR**

By

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*Dedicated to my father Qiaoyu Xu and my mother Yuling Chen*

## Abstract

Liver-enriched nuclear receptor (NR) proteins regulate the expression and activity of several pivotal hepatic biochemical pathways including the uptake, metabolism and excretion of cholesterol, bile acids, glucose, and xenobiotic compounds from the body. The pregnane x receptor (PXR, NR1I2) was first identified in 1998 as a member of the NR superfamily. Over the past decade, it has been well established that PXR functions as a master-regulator of xenobiotic- and drug-inducible expression and activity of numerous genes that encode key members of the phase I and phase II metabolic enzymes, as well as several membrane transporter proteins. In this way, activation of PXR serves as the principal defense mechanism defending the body from toxic insult. Similarly, the PXR protein also forms the molecular basis of an important class of drug-drug interactions in the clinical setting. Moreover, ligand-activated PXR negatively regulates inflammatory processes in both liver and intestine. An integrated model is emerging to reveal a key role for the post-translational modification of PXR in the selective suppression of gene expression, and is opening the door to the study of completely new modes of PXR-mediated gene regulation.

This dissertation contributes mainly to two key areas of PXR research: (1) Identification a novel PXR target gene- carboxylesterase 6 (Ces6); (2) a study of the SUMOylation and ubiquitination of PXR protein. The results presented in this dissertation were primarily obtained from mouse and cell-culture systems. Data presented here reveal that activation of the inflammatory response modulates the SUMOylation and ubiquitination status of ligand-bound PXR protein. The

SUMOylation and ubiquitination of the PXR protein functions to feedback-repress the inflammatory and xenobiotic responses, respectively. Taken together, the data represent a likely mechanism and provides initial molecular details for the connection between the PXR signaling pathway and inflammation. Studies on post-translational modification of PXR indicate how this protein is converted from a positive regulator in drug metabolism into a transcriptional repressor in inflammatory response. Finally, detailed protocols for purification of mammalian proteins necessary to perform *in vitro* SUMOylation reactions are presented. Taken together, the work presented in this dissertation contributes to understanding the interface between PXR, drug metabolism, and inflammation, which is expected to produce new opportunities for the development of novel therapeutic strategies.

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## List of Abbreviations

ABCA1: ATP Binding Cassette Transporter A1

ACBP: Acyl-CoA Binding Protein

ADR: Adverse Drug Reaction

ALLN: Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal

CAR: Constitutive Androstane Receptor

CAR-KO: Constitutive Androstane Receptor Knockout

CDK2: Cyclin-dependent Kinase 2

CES: Carboxylesterase

CHIP: Chromatin Immunoprecipitation

CRE: Cyclic AMP Response Element

CREB: Cyclic AMP Response Element Binding Protein

CYP: Cytochrome P450

DBD: DNA-binding Domain

DME: Drug Metabolizing Enzyme

ER: Estrogen Receptor

ERK: Extracellular Regulated Kinase

FOXA2: Forkhead box A2

FOXO1: Forkhead Box Transcription Factor O1

FXR: Farnesoid X Receptor

G6Pase: Glucose 6 Phosphatase

GR: Glucocorticoid Receptor

GST: Glutathione S Transferase

HA: Hydrolase A

HB: Hydrolase B

HNF4α: Hepatocyte Nuclear Factor 4 Alpha

HS: Hydrolase S

HSD17B11: 17-beta-Hydroxysteroid dehydrogenase type 11

IBD: Inflammatory Bowel Disease

IL: Interleukin

JNK: Jun-kinase

LBD: Ligand-binding Domain

LXR: Liver X Receptor

MAPK: Mitogen-activated Protein Kinase

MDR1: Multi-drug Resistance 1

MEK: Mitogen-activated Protein Kinase Kinase

MEKK1: Mitogen-activated Protein Kinase Kinase Kinase

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

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

NCoR: Nuclear Receptor Co-repressor

NFκB: Nuclear Transcription Factor Kappa B

NR: Nuclear Receptor

OATP2: Organic Ion Transporting Protein 2

RANGAP1: Ran GTPase Activating Protein 1

PB: Phenobarbital

PBREM: Phenobarbital Response Enhancer Module

PC2: Polycomb 2 Homolog

PCN: Pregnenolone 16α-carbonitrile

PDK4: Pyruvate dehydrogenase kinase, Isozyme 4

PEPCK: Phosphoenolpyruvate Carboxykinase

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

PIAS: Protein Inhibitors of Activated STAT

PKA: Cyclic-AMP-dependent Protein Kinase

PPAR: Perioxisome Proliferator Activated Receptor

PXR: Pregnane X Receptor

PXR-KO: Pregnane X Receptor Knockout

RANBP2: Ran Binding Protein 2

RAR: Retinoic Acid Receptor

RIF: Rifampicin

RXR: Retinoid X Receptor

SENP: Sentrin-specific Proteases

SUG1: Suppressor for Gal1

SULT: Sulfotransferase

SUMO: Small Ubiquitin-like Modifier

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

TNFα: Tumor Necrosis Factor Alpha

UGT: UDP Glucuronosyltransferase

XREM: Xenobiotic Responsive Enhancer Module

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## Chapter 1: Introduction

Nuclear receptor (NR) proteins comprise a large superfamily of ligand-activated transcription factors that are involved in diverse physiological, developmental and metabolic processes. They share certain amount of structural homology with a conserved N-terminal zinc-finger type DNA-binding domain (DBD) and a C-terminal ligand-binding domain (LBD) [1]. The pregnane x receptor (PXR, NR1I2) was first identified in 1998 as a member of the NR superfamily. In mammals, PXR is highly expressed in the major organs that are important in xenobiotic biotransformation including the liver and the intestine [2]. Over the past decade, it has been well established that PXR functions as a master-regulator of xenobiotic- and drug-inducible expression and activity of numerous genes that encode key members of the cytochrome P450 (CYP) drug-metabolizing enzymes in humans and rodents [3, 4]. PXR target genes also encode several glutathione *S*-transferase, sulfotransferase, and UDP-glucuronosyltransferase enzymes in the liver, as well as key hepatic drug transporter proteins, such as organic anion transporting polypeptide 2, multidrug resistance 1/P-glycoprotein, and multidrug resistance proteins 2 and 3 [5-10].

In addition to serving as a positive regulator in mediating drug metabolism and transport, clinical evidence has been accumulated to reveal the repressive function of the PXR protein. Recent studies indicate that ligand-mediated activation of PXR negatively regulates several key biochemical functions in the liver and intestine, including the synthesis of glucose and ketone bodies, β-oxidation, transport of lipids, as well as inflammatory processes.

The overall goal of this dissertation is to characterize the regulation of nuclear receptor PXR in drug metabolism and inflammation. Chapter 2 summarizes the current state of knowledge regarding NR-mediated regulation of carboxylesterase (CES) enzymes in mammals and highlights their importance in drug metabolism, drug-drug interactions and toxicology. Elucidation of the role of NR-mediated regulation of CES enzymes in liver and intestine will have a significant impact on rational drug design and the development of novel prodrugs, especially for patients on combination therapy.

Chapter 3 reviews PXR-mediated repression of gene expression programs underlying several pivotal physiological functions, including decreased capacities for gluconeogenesis, lipid metabolism, and inflammation. An integrated model is emerging that reveals a sophisticated interplay between ligand binding and the ubiquitination, 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.

In Chapter 4, microarray analysis is used to identify PXR target genes in duodenum in mice. We show that a gene encoding a member of the carboxylesterase2 (CES2) subtype of liver- and intestine-enriched CES enzymes, called Ces6, is induced after treatment with pregnenolone 16alpha-carbonitrile (PCN) in a PXR-dependent manner in duodenum and liver in mice. Treatment of mice with the CAR activator

1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) also induces expression of Ces6 in duodenum and liver in a CAR-dependent manner, whereas treatment with phenobarbital (PB) produces induction of Ces6 exclusively in liver. These data identify a key role for PXR and CAR in regulating the drug-inducible expression and activity of an important CES enzyme *in vivo*.

In Chapter 5, we identify PXR as a molecular target of ubiquitin. We show that ubiquitination of PXR is stimulated in cells by treatment with cyclic-AMP and activation of the MEKK1 signaling pathway, suggesting distinct regulation of PXR activity by metabolic- and inflammatory-mediated signaling. Interestingly, inhibition of the proteasomal degradation pathway and increased ubiquitination of PXR represses rifampicin-inducible PXR transactivation capacity in an engineered PXR reporter gene assays. Taken together, this novel data provides a plausible and testable hypothesis for how inflammatory- and cyclic AMP/PKA-mediated signaling pathways selectively repress the drug-inducible expression and activity of hepatic drug metabolizing and drug transporter activities in the liver and the intestine.

In Chapter 6, we show that activation of the inflammatory response in hepatocytes strongly modulates SUMOylation of ligand-bound PXR. We provide evidence that the SUMOylated PXR contains SUMO3 chains, and feedback represses the immune response in hepatocytes. This information represents the first step in developing novel pharmaceutical strategies to treat inflammatory liver disease and prevent adverse drug reactions in patients experiencing acute or systemic inflammation. These studies also provide a molecular rationale for constructing a novel paradigm that uniquely defines

the molecular basis of the interface between PXR-mediated gene activation, drug metabolism, and inflammation.

In Chapter 7, we present detailed protocols for bacterial expression, isolation, and purification of mammalian proteins necessary to perform *in vitro* SUMOylation reactions, namely the SUMO E1 enzyme (AOS1/UBA2 heterodimer), Ubc9, and SUMO1, SUMO2, and SUMO3. Detailed methods for performing *in vitro* SUMOylation assay by SUMO1, SUMO2, and SUMO3 using RanGap1 as substrate are also described. SUMOylation is a relatively new protein modification and methods for research are still being developed. The protocols described in Chapter 7 are useful for research of this post-translational modification.

Taking together, the work presented in this dissertation contributes mainly to two key areas of PXR research: (1) Identification of carboxylesterase6 as a novel PXR target gene in liver and intestine tissues further characterizes the well-established role for PXR in drug metabolism; (2) Revealing the SUMOylation and ubiquitination of ligand-bound PXR provides a possible molecular mechanism connecting PXR and inflammation. The identification of novel ligands and target genes continues to be an important aspect of PXR research. Studies on post-translational modification of PXR contribute to the investigation of how PXR is converted from a positive regulator in drug metabolism into a transcriptional repressor in inflammatory response. Understanding of the interface between PXR, drug metabolism, and inflammation is critical for the development of safe and effective therapeutic strategies.

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# Chapter 2: Nuclear Receptor-mediated Regulation of Carboxylesterase Expression and Activity

## *2.1 An Introduction to Carboxylesterase*

### *2.1.1 Classification of Carboxylesterase*

In 1953, Aldridge classified esterase enzymes in rabbit, rat, and horse serum based upon the nature of their interaction with organophosphates [1]. Esterases that were unaffected by organophosphates and degraded the compounds were classified as A-esterases, whereas esterases that were inhibited by organophosphates were classified as B-esterases. Studies by Bergmann et al. revealed the presence of a third group of esterases (the C-esterases) that were not affected by and did not interact with organophosphates at all [2]. Using this classification scheme, the superfamily of carboxylesterase (CES) enzymes belong to the B-esterase group. Several attempts have been made to classify the CES enzymes. Walker and Mentlein et al., attempted to classify CES enzymes on the basis of their substrate specificity [3, 4]. However, this classification scheme was ambiguous because of the broad and overlapping substrate specificity of CES enzymes. In 1998, Satoh and Hosokawa originally proposed a novel classification scheme of CES enzymes across species that was based upon the extent of amino acid homology and substrate selectivity [5]. This scheme classified the known CES enzymes into four main groups (CES 1-4), and several additional subgroups. More recently, the same authors have used this same scheme to show that there are five groups of CES enzymes (CES 1-5), and revealed that the majority of identified CES enzymes belong to either the CES 1 or CES 2 sub-family [6].

Importantly, it is now known that the CES-1 and CES-2 sub-families are the major source of carboxylesterase enzymatic activity in liver and intestine tissues that participate in the hydrolysis of drugs and xenobiotics in mammals [6].

### *2.1.2 Function of Carboxylesterase Enzymes*

The CES family of enzymes is a key participant in the phase-I drug metabolism process, catalyzing the hydrolysis of a wide range of ester- and amide-containing compounds. Of particular clinical relevance, these enzymes participate in the biotransformation of numerous drugs and prodrugs including the anti-platelet drugs aspirin and clopidogrel [7], the angiotensin-converting enzyme inhibitors delapril, imidapril, and temocapril [8], the anti-tumor drugs irinotecan and pentyl PABC-doxaz [9, 10], the narcotics cocaine and heroin [11], and the anti-influenza prodrug oseltamivir [12, 13]. The CES family of enzymes is involved in the detoxification of environmental toxicants, such as pyrethoids, a major class of insecticides used worldwide and extensively in the United States [14]. CES enzymes also play a role in the conversion of pro-carcinogens into carcinogens. For example, vinyl acetate, which is used in the paint, adhesive, and paper-board industry, is metabolized by CES enzymes into acetaldehyde in the liver. Acetaldehyde subsequently binds to DNA and proteins eventually leading to nasal tumor formation in rodents [15]. Numerous endogenous compounds are substrates for CES enzymes including palmitoyl-coenzyme A, short- and long-chain acyl-glycerols, as well as medium- and long-chain acylcarnitines [5, 16]. Because a large number of clinically used drugs and

prodrugs are metabolized by CES enzymes, it is important to clarify the structure, substrate selectivity, tissue distribution, and species specificity of CES enzymes.

### *2.1.3 Structure of CES Enzymes*

The crystal structure of human carboxylesterase 1 (hCE-1) was determined in 2003. The enzyme is comprised of three structural domains: a central catalytic domain, an $\alpha/\beta$ domain, and a regulatory domain. The central catalytic domain contains the serine hydrolase catalytic triad at the base of the active site gorge, whereas the regulatory domain contains the low-affinity surface ligand-binding Z-site [17, 18]. The CES enzymes are localized in the endoplasmic reticulum and cytosol of many tissues, but are highly enriched in liver and intestine [6, 19]. It has been determined that an 18-amino acid N-terminal hydrophobic signal peptide is responsible for the localization of these proteins to the endoplasmic reticulum [20], whereas enzymatic activity is lost by removing the N-terminal domain. The His-X-Glu-Leu (HXEL) sequence present at the C-terminal, which can bind with KDEL receptor, is essential for retention of the protein in the luminal site of the endoplasmic reticulum.

### *2.1.4 Substrate Selectivity of CES Enzymes*

Amino acid sequence homology between human carboxylesterase 1 (hCE-1), a member of CES 1 family, and human carboxylesterase 2 (hCE-2), which belongs to CES 2 family is 48% [10]. However, the substrate selectivity of these two enzymes is different. The hCE-1 enzyme mainly hydrolyzes substrates with small alcohol groups and large acyl groups, such as cocaine (methyl ester), meperidine, and delapril. In contrast to hCE-1, the hCE-2 enzyme efficiently hydrolyzes compounds with large

alcohol groups and relatively smaller carboxylate groups, such as 4-methylumbelliferyl acetate, heroin, and 6-acetylmorphine [21].

### *2.1.5 Tissue Distribution of CES Enzymes*

The expression of CES enzymes is ubiquitous in mammals. Among various tissues of mammals, the highest hydrolase activity is present in liver [22]. In addition to liver, CES enzymes are also detected in small intestine, kidney, and lung [6]. The hCE-1 enzyme is highly expressed in the liver, and also detected in macrophages, human lung epithelia, heart, and testis [23]. The hCE-2 enzyme is found in the small intestine, colon, kidney, liver, heart, brain, and testis [19, 24]. Although these two enzymes are present in various tissues, hCE-1 and hCE-2 contribute predominantly to the hydrolase activity of liver and small intestine, respectively. It has also been shown that CES enzymes exhibit species differences. For example, Li et al demonstrated that human plasma contains no CES enzyme activity, in contrast, the mouse, rat, rabbit, horse, cat, and tiger all have high levels of plasma CES enzymes [25]. However, in humans it is likely that serum butyrylcholinesterase and paraoxonase enzymes perform analogous functions to the CES enzymes found in serum from these other species.

## *2.2 PXR and CAR, Two Xenobiotic-Sensing NRs*

As CES enzymes play very important roles in drug metabolism, their expression levels are tightly controlled by the NR proteins pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3). NR proteins comprise a large superfamily of ligand-activated transcription factors that are involved in diverse physiological, developmental, and metabolic processes. They are characterized by a

conserved N-terminal zinc-finger type DNA-binding domain and a C-terminal ligand-binding domain [26]. The PXR and CAR proteins are two closely related members of this superfamily. Both of these proteins function as ligand-activated transcription factors by interacting with the retinoid-x-receptor-alpha (RXRα, NR2B1) on response elements located in the control regions of specific genes that they regulate.

PXR functions as a master-regulator of xenobiotic- and drug-inducible cytochrome P450 (CYP) gene expression in liver and it is now well established that PXR regulates the drug-inducible expression and activity of numerous genes that encode key members of the CYP3A, CYP2B and CYP2C subfamily of drug-metabolizing enzymes in humans and rodents [27, 28]. PXR also regulates the drug-inducible expression of other genes whose gene products are involved in the metabolism of xenobiotic compounds including glutathione *S*-transferase, sulfotransferase, and UDP-glucuronosyltransferase enzymes in liver [29-32]. Moreover, additional PXR-target genes encode key hepatic drug transporter proteins such as organic anion transporting polypeptide 2, multidrug resistance 1/P-glycoprotein, and multidrug resistance proteins 2 and 3 [33-35].

Similar to PXR, the NR superfamily member CAR is also recognized as a xenobiotic-sensing NR mainly expressed in hepatic tissue. It was originally demonstrated to regulate the phenobarbital-inducible expression of several genes encoding important members of the CYP2B subfamily of enzymes [36]. CAR has since been shown to regulate the expression and activity of a number of phase-I and phase-II metabolic enzymes, as well as the expression and activity of numerous

important membrane transporter proteins involved in the metabolism and elimination of xenobiotics [37]. It has been demonstrated that PXR and CAR share distinct but overlapping sets of target genes involved in drug and xenobiotic metabolism, often through shared NR-response elements. For instance, PXR can regulate CYP2B genes through recognition of the Phenobarbital-response element (PBREM), whereas CAR is also found to activate gene expression through the xenobiotic response element (XREM) in the upstream promoter of the CYP3A4 gene in humans [29, 38, 39]. Because PXR and CAR are activated by a myriad of xenobiotic compounds and regulate the expression of numerous genes involved in drug and xenobiotic metabolism, the activation of these two receptors serves as a principal defense mechanism defending the body from toxic insult. In this way, activation of PXR and CAR by xenobiotic compounds and drugs coordinately regulates the expression and activity of functionally linked metabolic enzymes and membrane-bound transporter proteins to increase the elimination of potentially toxic compounds from the body [27, 32, 39-41]. Additionally, these two transcription factors form the molecular basis of an important class of drug-drug interactions in the clinical setting. PXR and CAR-mediated gene activation by one drug increases the metabolism and elimination of a myriad of other co-administered drugs from the body.

### *2.3 Regulation of CES Enzymes by PXR and CAR*

#### *2.3.1 Regulation by PXR*

The hCE-1 and hCE-2 genes encode the two major forms of human liver microsomal carboxylesterase enzymes. Studies by Zhu et al. show the involvement of

PXR in regulating the expression and activity of these two enzymes [42]. Exposure of primary cultures of human hepatocytes to micromolar concentrations of dexamethasone induces hCE-1 and hCE-2 protein expression in a concentration-dependent manner. Treatment of human hepatocytes with ten micromolar rifampicin, the prototypical human PXR-activating compound, causes moderate induction of hCE-1 and hCE-2 gene expression [43]. In addition to dexamethasone, treatment of cultured human hepatocytes with 8-methoxypsoralen, which is a prototypical photochemotherapeutic drug, increases hCE-2 gene expression [13]. Moreover, knockdown of PXR using si-RNA technology decreases hCE-2 mRNA levels, whereas over-expression of the PXR protein significantly increases hCE-2 expression at both the messenger RNA and protein levels.

PXR also induces the expression of CES enzymes in rodents. In rats, the best characterized carboxylesterase enzymes include hydrolase A, B and S (HA, HB, HS). Co-transfection of PXR stimulates the promoter activity of HB and HS in response to dexamethasone at micromolar concentrations [43]. Tully et al. characterized the effects of triazole fungicides in SD rats using microarray analysis [31]. Gene expression profiling of liver shows induction of Ces2 is produced by four triazole fungicides, and is likely dependent on PXR/CAR-mediated gene activation pathways. A similar study by Goetz et al., utilized gene expression profiling of the liver of CD-1 mice treated with four triazole fungicides. Expression of the Ces2 gene is induced by three triazole fungicides, suggesting involvement of PXR/CAR-regulated pathways in triazole metabolism and perhaps toxicity [44]. Earlier research by Rosenfeld et al.,

indicates that over-expression of a constitutively active form of human PXR in mouse liver has a positive effect on the expression of mouse genes encoding Ces2 and Ces3 enzymes in liver [45].

The mouse Ces6 gene was first identified in 2004 and encodes a protein of 558 amino acid residues in length that functions to hydrolyze select pyrethroid compounds [46]. Recently, our lab has demonstrated that the Ces6 gene represents a likely PXR-target gene in mouse liver and small intestine [47]. By exploiting the PXR knockout mouse model, we reveal that induction of Ces6 messenger RNA and protein by pregnenalone 16α-carbonitrile (PCN), a well known rodent PXR activator, is PXR-dependent in both mouse liver and intestine.

### *2.3.2 Regulation by CAR*

Compared to PXR, relatively little is known about the regulation of drug-inducible CES gene expression by CAR activation in any species or tissue. Historical reports indicate that treatment of rats with phenobarbital (PB) increases CES expression in liver tissue [48]. Studies from Xu et al. show that Ces6 represents a CAR-target gene in mouse liver and small intestine [24]. It is interesting to note that in small intestine, the expression of Ces6 is exclusively regulated by 1,4-Bis[2-(3,5-dichloropyridyloxy)]benzene (TCPOBOP) but not by PB, both of which are CAR activators. These data suggest that there may be differences in the bioavailability of PB and TCPOBOP, or perhaps the differences in the mode of CAR activation by these two ligands in small intestine are responsible for the absence of Ces6 gene activation by PB. Moreover, TCPOBOP is a much more potent and

efficacious activator of rodent CAR, thus is a much more effective chemical to use in rodent studies.

### *2.3.3 Coordinate Regulation of Gene Expression by PXR and CAR*

Using ChIP-seqeuencing analysis of control and PCN-treated mouse livers, we observed constitutive PXR-binding to three enhancer elements located in the upstream region of the mouse *Ces6* gene under physiological conditions (Figure 2-1, top panel), which are approximately 84bp (site 1), 1796bp (site 2), and 2340bp (site 3) upstream of the transcription start site of *Ces6*. Most interestingly, treatment with the mouse PXR agonist PCN produces an approximately 2-fold overall increase in PXR binding to all the three sites, particularly to the second site (site 2), which binds to PXR with the highest affinity. In addition, a new PXR binding site occurs further upstream (-2772bp) with moderate fold-enrichment (average value = 40) (Figure 2-1, bottom

**Figure 2-1**

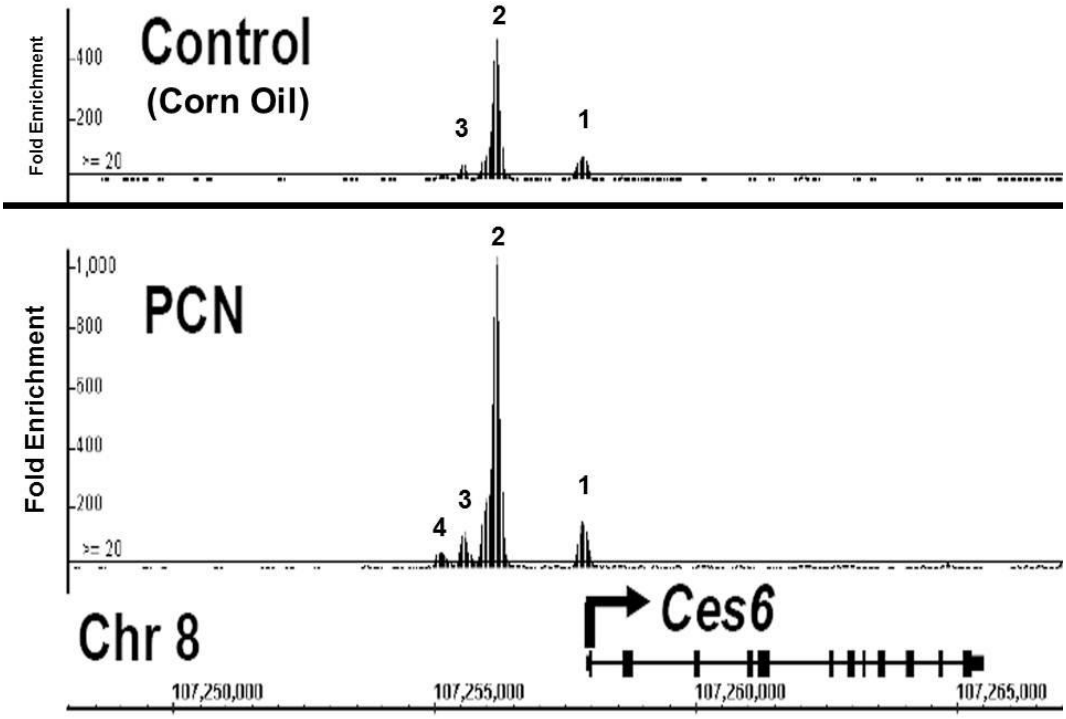


**Figure 2-1. PXR protein binds 'site 2' *in vivo* with the highest affinity.** Total crosslinked chromatin was subject to immunoprecipitation with an anti-PXR antibody (n=2). The anti-PXR enriched immunoprecipitated chromatin was subjected to high-throughput DNA sequence analysis and mapped back to the mouse genome. The exact locations of enriched fragments along with their proximities to the annotated Ces6 gene were then determined. A. Control mouse liver treated i.p. with corn oil only. B. Mouse liver that were treated with PCN (200 mg/kg, i.p. in corn oil). ChIP-sequencing raw data were normalized by the sequencing depths. Significant PXR binding is determined based on a threshold value of 20-fold enrichment based upon the false discovery rate. Data are visualized by the integrated genome browser and are expressed as fold-enrichment.

panel). Close examination of the DNA sequences that constitute site 2 reveals a cluster of likely NR-response elements located within 70 base pairs of each other and these are depicted in figure 2-2. Using two oligonucleotides, designated as 'long' and 'short', derived from this DNA sequence we performed electrophoretic mobility-shift analysis and show that both CAR/RXRα and PXR/RXRα protein complexes bind directly to these putative response elements (Figure 2-3). Importantly, competition-binding using an oligonucleotide that comprises the prototypical shared PXR/CAR response element, an everted repeat spaced by 6 nucleotides (ER6) derived from the well-characterized promoter of the CYP3A4 gene, shows that binding to the putative Ces6 response elements is specific. Conversely, a mutant form of the same oligonucleotide (mtER6) did not compete for binding, whereas the homologous oligonucleotides comprising the 'long' and 'short' experimental oligonucleotides compete well for binding of both the CAR/RXRα and PXR/RXRα protein complexes. Hence, the PXR and CAR NR superfamily members play direct and competitive roles in regulating the drug-inducible expression and activity of an important liver- and intestine-enriched mouse CES enzyme. Together with numerous other drug-metabolizing enzymes and drug transporter proteins in liver and intestine, PXR and CAR regulate the expression and activity of key CES enzymes that coordinately determine the pharmacokinetic and pharmacodynamic properties of numerous clinically prescribed and xenobiotic compounds in vivo in liver and intestine. Taken together, the data lead to a model in which drug-inducible activation of intestinal CES

**Figure 2-2**

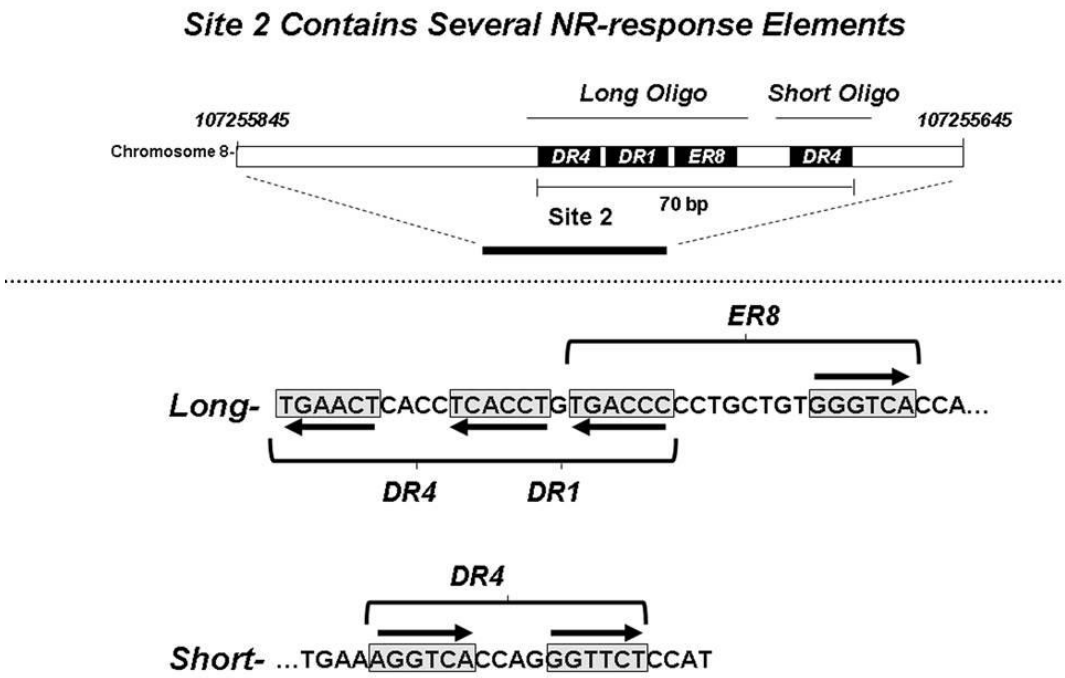


**Figure 2-2. Site 2 contains several NR-response elements.** DNA sequence of site 2 was examined using Nubiscan (http://www.nubiscan.unibas.ch/) and NHR scan (http://asp.ii.uib.no:8090/cgi-bin/NHR-scan/nhr_scan.cgi) websites to identify all NR half-sites and predict putative PXR- and CAR-binding sites. The 'long' and 'short' double-stranded oligonucleotides encompassing the putative-binding sites were generated.

**Figure 2-3**

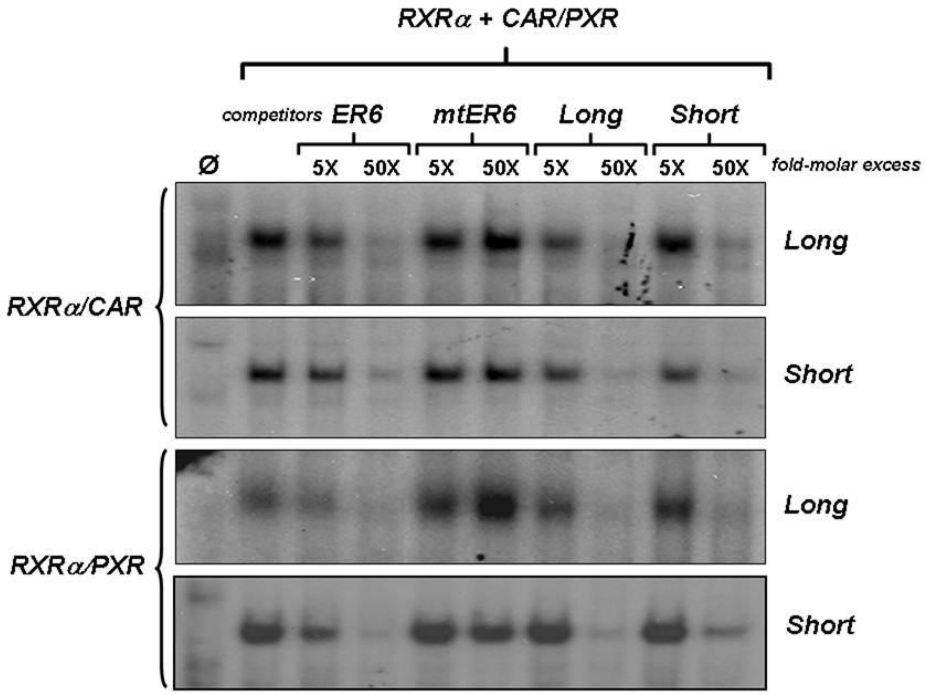


**Figure 2-3. EMSA using the 'long' and 'short' double-stranded radiolabeled oligonucleotides.** EMSA analysis was performed using the radiolabeled double-stranded 'long' and 'short' oligonucleotides using standard methods as described previously. The shifted complex was effectively competed with the non-radiolabeled and prototypical shared PXR/CAR response element derived from the CYP3A4 gene that is called ER6, the 'long' and the 'short' oligonucleotides as indicated. However, the mutant ER6 (mtER6) did not compete for binding to any detectable degree. Thus, binding of RXRα/CAR and RXRα/PXR complexes to these two oligonucleotides that are derived from the Ces6 upstream promoter was specific.

activity in intestine would be expected to increase the conversion of prodrugs to the active form of the drug, thereby increasing transport to portal vein and liver (Figure 2-4). In the liver, high levels of cytochrome P450 and CES activity would be expected to further increase metabolism of co-administered drugs, thereby leading to increased prospects for drug-drug interaction in patients on combination therapy. Moreover, activation of these pathways by PXR and CAR would be expected to increase the conversion of pro-carcinogens into carcinogenic compounds in these tissues.

### *2.4 Other Nuclear Receptors Regulate CES Enzymes*

In addition to PXR and CAR, CES enzymes are also regulated by other NR proteins, such as hepatocyte nuclear factor-4α (HNF-4α, NR2A1), peroxisome proliferator-activated receptor α (PPARα, NR1C1), and glucocorticoid receptor (GR, NR3C1). HNF-4α is mainly expressed in liver, intestine, pancreas and kidney, and is critical for transcriptional regulation of many genes in liver, such as Cyp7a1, CAR, and genes involved in the control of lipid homeostasis, glucose transport and glycolysis [49-52]. HNF-4α has also been implicated in the regulation of mouse Ces2 gene transcription. In the same study, bile acids are shown to repress expression of mCES2 by inhibiting the HNF-4α-mediated transactivation of the mCES2 gene promoter [53].

Proxisome proliferator-activated receptors (PPARs) are mainly involved in lipid and glucose homeostasis, control of inflammation and wound healing, and regulation of food intake and body weight [54, 55]. However, there appears to be a connection between PPARs and hepatic CES gene expression in rodents as well. The PPARα

**Figure 2-4**

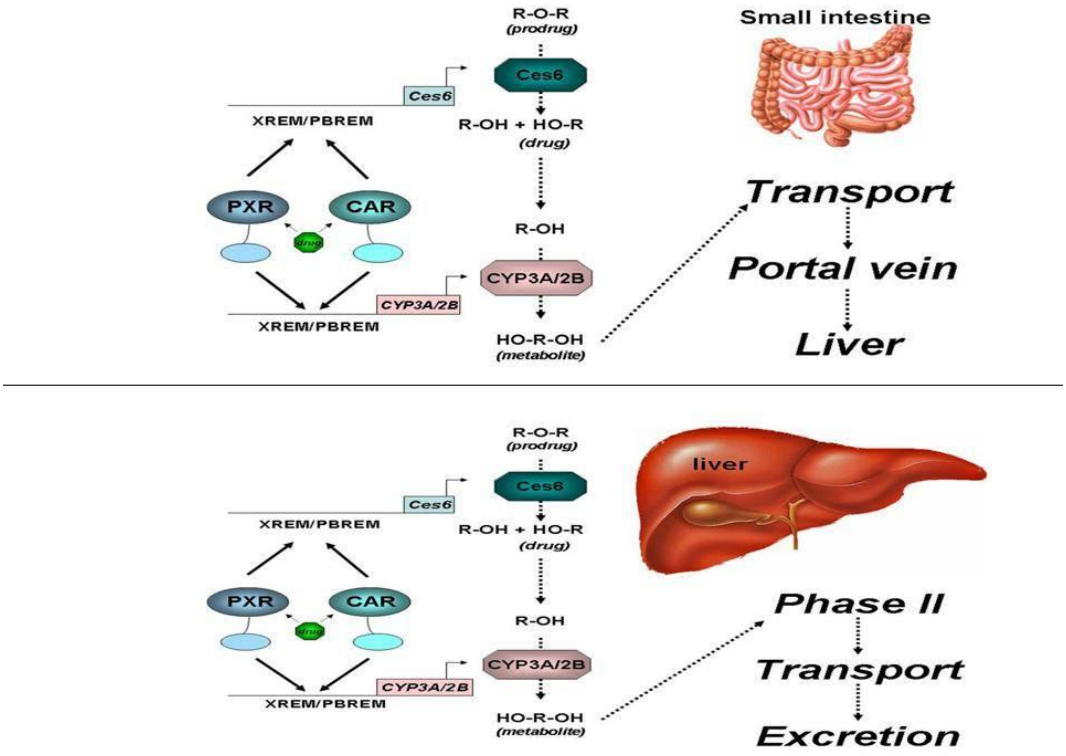


**Figure 2-4. Model of the significance of PXR-and CAR-mediated gene activation in liver and intestine.** A. Activation of PXR in intestine produces elevated levels of Ces6 and Cyp3A activity. This would be expected to accelerate conversion of prodrug to active drug and increase uptake into the portal circulation. B. The liver would then mediated further uptake metabolism and excretion into bile and elimination in feces or back into blood for eventual elimination through the kidney and in urine.

protein, one of the three subtypes of PPARs, is predominantly expressed in tissues with a high oxidative capacity such as heart and liver. Research by Poole et al. showed that exposure to peroxisome proliferators, strong activators of PPARα in liver, leads to down-regulation of the expression of CES family members. The alteration in CES expression is dependent on the PPARα protein in mouse [56].

***2.5 Conclusions.***

NRs are key regulators of many drug metabolizing enzymes that play diverse roles in xenobiotic and endobiotic metabolism. This review summarizes the evidence that several key NR proteins, including PXR, CAR, HNF-4α, PPARα, and GR, are involved in the regulation of CES enzymes. Because CES enzymes are one of the major determinants of the metabolism and disposition of numerous prodrugs through their actions in liver and small intestine, elucidating the mechanism governing the regulation of CES enzyme expression and activity by NR proteins will have a significant impact on rational drug design and the future development of prodrugs.

It is well known that activation of NRs, such as PXR and CAR, coordinately regulates the expression and activity of numerous drug-metabolizing enzymes as well as multiple drug transporter proteins. Not only can this coordinated regulation protect cells from toxic insult, but it also represents the molecular basis for an important class of drug-drug interactions in clinical settings. For example in multi-drug therapy, if one drug activates PXR and/or CAR, and the other is administered as a prodrug that is metabolized and eliminated by PXR/CAR-target genes, the resulting increased biotransformation of the prodrug into an active drug would probably lead to markedly

discrepant pharmacological activities and pharmacokinetic behavior, or even serious and toxic side effects. As is the case with several anticancer drugs, there is an emerging role for PXR and CAR in regulating CES enzymes that exert an important effect on the hydrolytic biotransformation of a number of clinically used drugs and prodrugs. Additional evidence is emerging which points to a key role for PXR in regulating blood-brain barrier permeability in response to drug treatment [57, 58], and also in modulation of multi-drug resistance and estrogen sensitivity in certain breast cancers [59]. Further elucidation of the role of the PXR protein in these clinically significant areas will likely produce important information that could be exploited as novel targets for cancer treatments.

Numerous classes of xenobiotic compounds activate either PXR or CAR including numerous clinically prescribed drugs, active compounds in popular herbal remedies, several prodrugs that are anti-cancer agents, drug metabolites, and the list is growing. Activation of these receptors by anti-cancer drugs would be expected to have profound impact on the pharmacokinetics of drug metabolism in patients taking prodrugs activated by CES and eliminated by the action of the CYP3A4 enzyme in liver. Moreover, both PXR and CAR activities appear to be modulated through alterations in post-translational modifications such as phosphorylation [60-63]. It is likely that these and other key signaling pathways are altered in patients experiencing disease states such as inflammation or diabetes. There is increasing recognition that key drug metabolism pathways are under metabolic control, and are altered in patients who are administered drugs while fasting or are cachectic. Because the activity of PXR and

CAR proteins also appear to be under metabolic control [61, 63], these two transcription factors are likely to be, in part, responsible for such alterations. If true, this would have enormous implications in the field of drug-drug interactions in the most ill cancer patients that are undergoing polytherapy with simultaneous pharmacological interventions who are experiencing cachexia. More research needs to be conducted into the possible metabolic control of PXR and CAR activity.

The observation that treatment with GR and PPARα agonists produces repression of CES gene expression in rodent models could also have a significant impact on patient care. If the same is true for humans, it would be expected that the numerous clinically prescribed medications and newly discovered drug candidates that work through GR and PPARα would suppress the biotransformation of the anticancer prodrugs that are targeted for biotransformation by CES enzymes. Obviously, more research is necessary to clearly elucidate differences in the regulation of drug-inducible expression and activity of CES enzymes in liver and intestine across species. This is particularly important because of the use of rodent models to determine drug efficacy and drug toxicity screening by the pharmaceutical industry. Thus, continued research using knockout mice, "humanized" mouse models, and human cell-based model systems will undoubtedly contribute significant knowledge that will elucidate the molecular mechanisms governing the regulation of CES enzyme expression and drug metabolism activity by these important metabolic enzymes. This thrust of research highlights the importance of monitoring the ratio and efficacy of the conversion of prodrug into active drug in patients receiving novel combination therapies.

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# Chapter 3: Post-translational Modification of Pregnane X Receptor

## *3.1 An Introduction to Pregnane X Receptor*

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 RXRα. 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].

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 pregnenalone 16α-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 $EC_{50}$ values of steroids that activate PXR are low-micromolar,

generally two to three orders of magnitude higher than concentrations found in circulating plasma [5, 6].

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 aminolevulonic 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].

### *3.2 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 3-1). 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.

### *3.2.1 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.

**Figure 3-1**

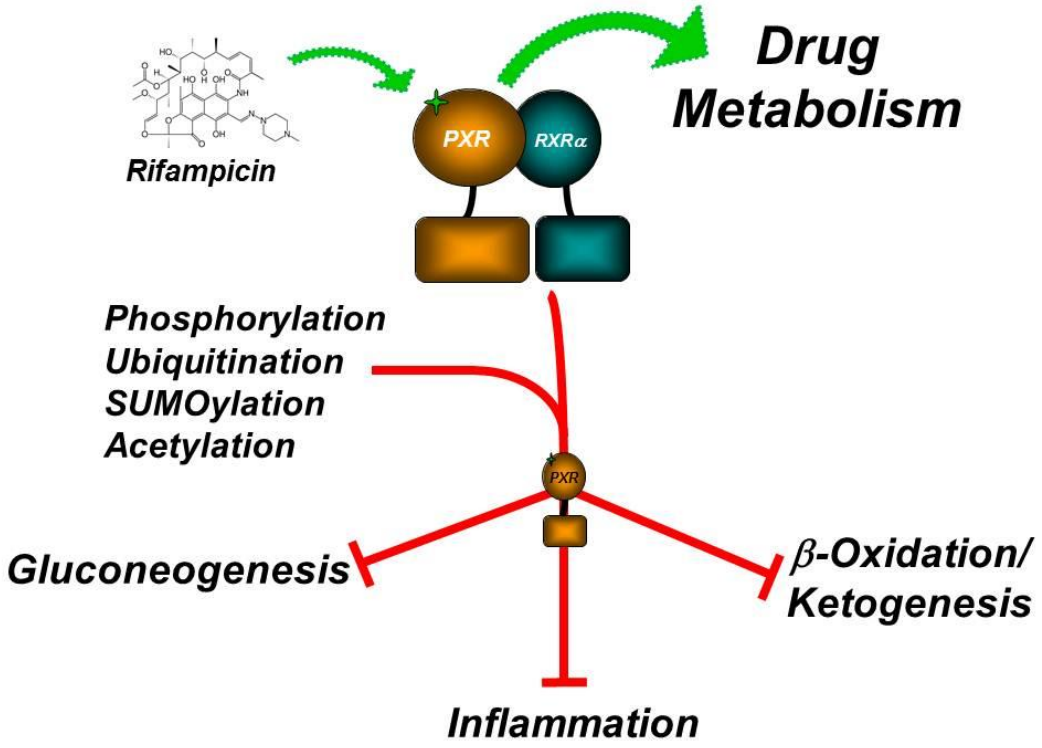


**Figure 3-1. Negative Regulation Roles of PXR.** PXR was originally characterized for its role in xenobiotic and endobiotic detoxification. However, recent evidence has described a role for PXR in glucose and lipid homeostasis, as well as repression of inflammatory programs of gene expression. A central role for post-translational modification of PXR is hypothesized to selectively repress biochemical pathways in liver and intestine.

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-1α) is induced by glucagon and coactivates hepatocytes nuclear factor 4α (HNF-4α)-mediated transcription of G6Pase and PEPCK. Ligand-activated PXR dissociates PGC-1α from HNF-4α through a direct competition/squelching mechanism, thereby repressing the transcription of PEPCK and G6Pase [18]. Since PGC-1α is also a co-factor for CREB- and FOXO1-mediated expression of gluconeogenic-target genes, a similar mechanism implicating sequestration of PGC-1α 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 gluconeogenisis such as FOXO1, CREB, HNF4α, and PGC-1α.

### *3.2.2 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-4α directly regulates expression of carnitine palmitoyltransferase 1A [31]. It has been demonstrated that PXR interferes with HNF-4α signaling by targeting PGC-1α and producing a squelching effect [18]. Since HNF-4α and PGC-1α 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.

### *3.2.3 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-κB), thus providing a potential molecular mechanism that links xenobiotic metabolism and inflammation [35]. Activation of PXR by Rif suppresses the expression of typical NF-κB target-genes such as cyclooxygenase-2, tumor necrosis factor α (TNFα), intercellular adhesion molecule-1 and several interleukins [36]. Conversely, NF-κB activation by lipopolysaccharide and TNFα results in the suppression of CYP3A activity through interactions of NF-κB with the PXR-retinoid-x-receptor complex [37]. Furthermore, hepatocytes derived from PXR-null mice have elevated NF-κB target-gene expression compared to hepatocytes from wild-type mice. The PXR-null mice also exhibit heightened signs of inflammation in their liver and small bowel [36, 38]. This could

be due to the loss of negative regulation of NF-κB 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.

### *3.3 Post-translational Modification of PXR*

#### *3.3.1 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 3-2A). As expected, detection of ubiquitinated PXR protein is dramatically increased in response to pharmacological inhibition of 26S proteasome activity with MG132 (Figure 3-2B, lane 4). Interestingly, forced activation of the PKA signaling pathway selectively increases the ubiquitination of PXR (Figure 3-2B, lane 6, note the asterisks). Notably, pharmacological inhibition of the proteosomal degradation pathway abolishes PXR transactivation of the CYP3A4 promoter in reporter gene transfected CV-1 cells (Figure 3-2C). 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 (PPARγ, NR1C3) and liver-x receptor alpha/beta (LXRα/β, NR1H3/NR1H2) [42-44]. The interaction between PXR and the ubiquitin signaling pathway appears to be relatively complex and warrants further investigation.

### *3.3.2 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

**Figure 3-2A**

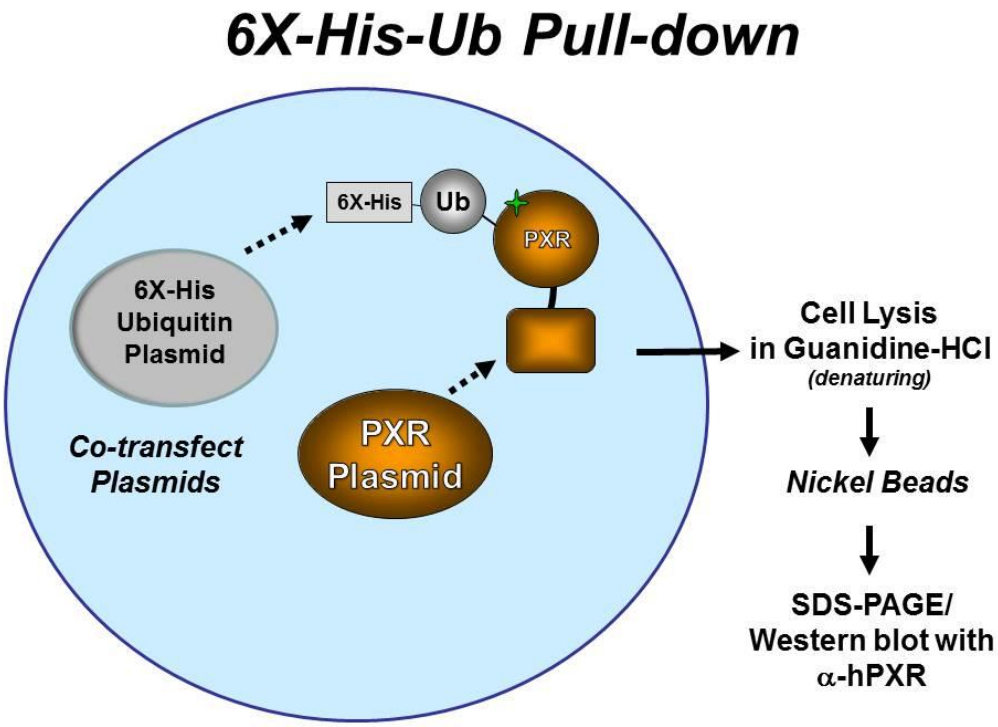


**Figure 3-2. Ubiquitination of PXR.** (A) Mammalian expression vectors encoding affinity-tagged (6X-histidine) ubiquitin and human PXR are introduced into cultured HeLa cells using lipofectamin as described [36]. Twenty-four hours post-transfection, cells were treated with Rif (10 μM), MG132 (25 μM), 8-Bromo cyclic AMP (0.5 mM, 8-Br-cAMP), or 8-bromo cyclic GMP (0.5 mM, 8-Br-cGMP) for an additional 24 hours. Whole-cell lysates were generated using denaturing conditions as described [64].

**Figure 3-2B**

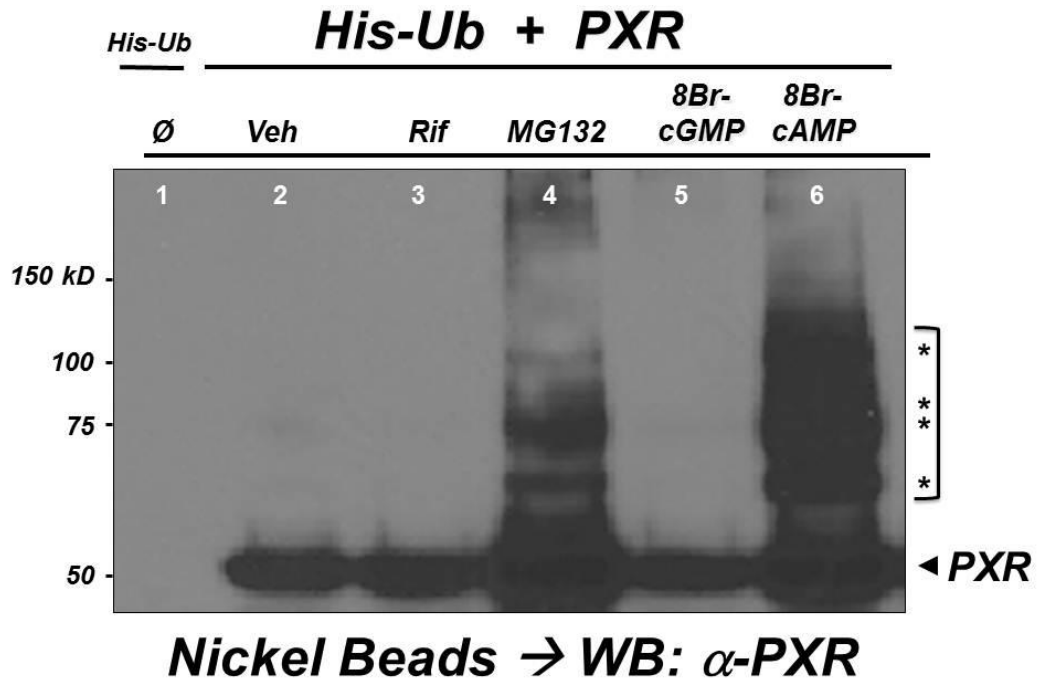


**Figure 3-2. Ubiquitination of PXR.** (B) Western blotting was performed using a monoclonal antibody specific for human PXR (sc-48340, Santa Cruz).

**Figure 3-2C**

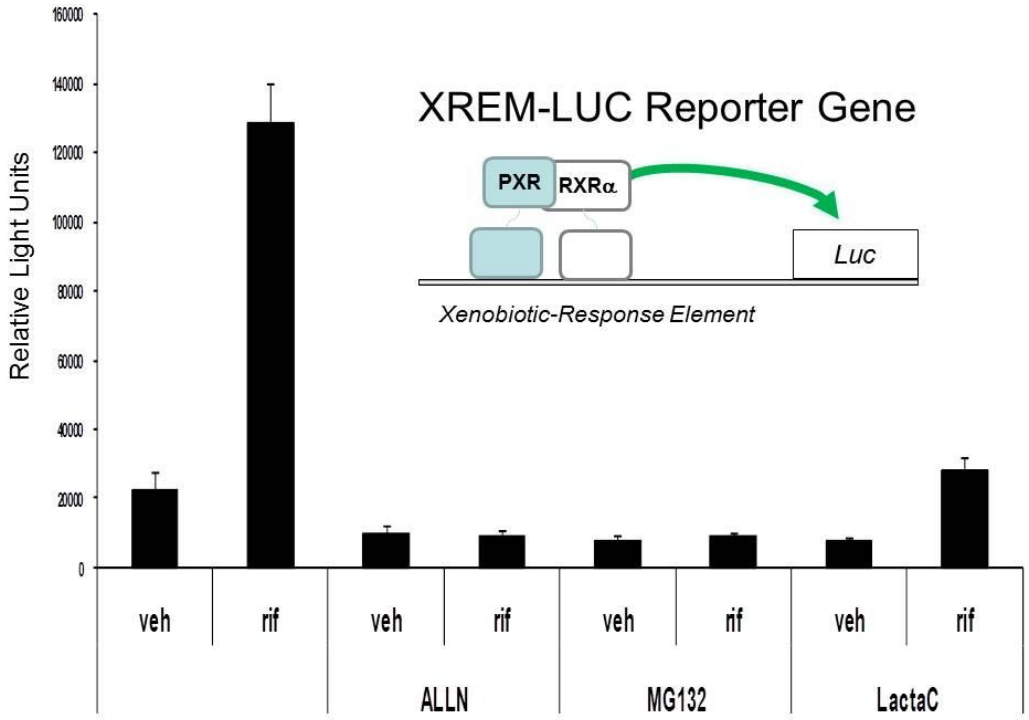


**Figure 3-2. Ubiquitination of PXR.** (C) The PXR-dependent XREM-LUC reporter gene [2] was transfected together with an expression vector encoding human PXR. 24 hours post-transfection, cells were treated with vehicle, Rif (10mM), ALLN (100 μM), MG132 (25 μM), or lactacystin (10 μM) for an additional 24 hours. The data are reported as relative light units and represent the mean of assays performed in triplicate ± SE.

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.

### *3.3.3 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 3-3A. 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 3-3B.

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 diglycil 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 3-3C. 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 3-3C, Step1). 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 3-3C, 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 susbstrates 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 3-3D, compare left panel and middle panel).

When SUMO-X is substituted for SUMO-3, we observe a similar result (Figure 3-3D, 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 3-3, note asterisks). This is highly reminiscent of NR PPARγ 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 PPARγ and its ability to transrepress NF-kB activity [43].

**Figure 3-3A**

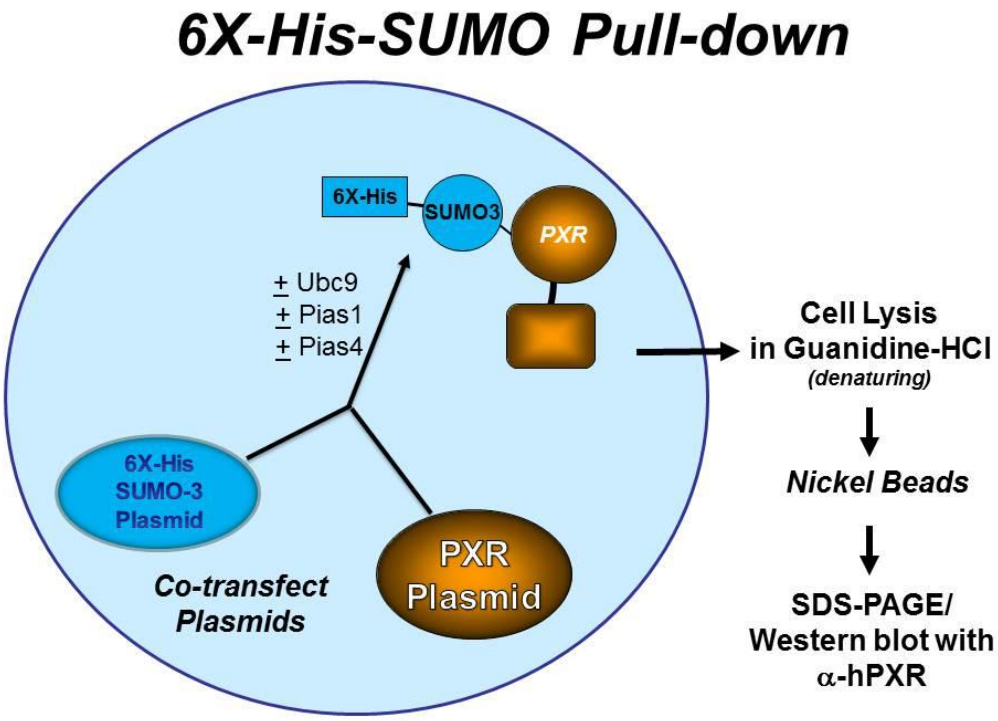


**Figure 3-3. SUMOylation of PXR.** (A) Mammalian expression vectors encoding affinity-tagged (6X-histindine) SUMO-3 and human PXR are introduced into cultured HeLa cells in the presence and absence of E2 ligase (Ubc9), and the E3 ligase enzymes PIAS1 or PIAS4. 48 hours post-transfection, whole-cell lysates were generated as described [64].

**Figure 3-3B**

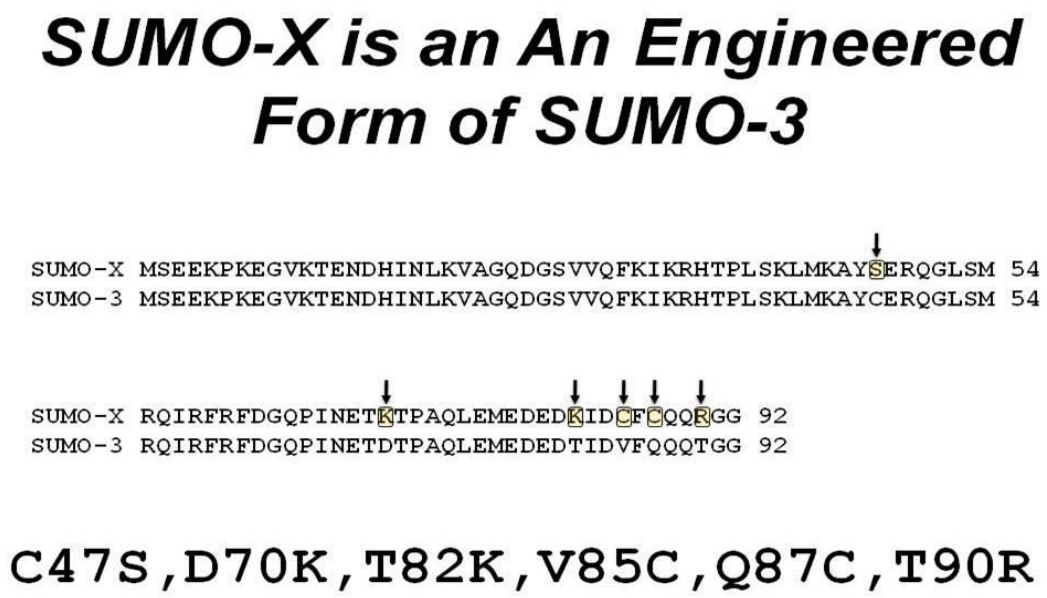


**Figure 3-3. SUMOylation of PXR.** (B) The SUMO-X protein incorporates several key point mutations to allow non-biased enrichment and identification of SUMOylated PXR peptides when expressed together with substrates in vitro, in cultured cells, or in live animals.

**Figure 3-3C**

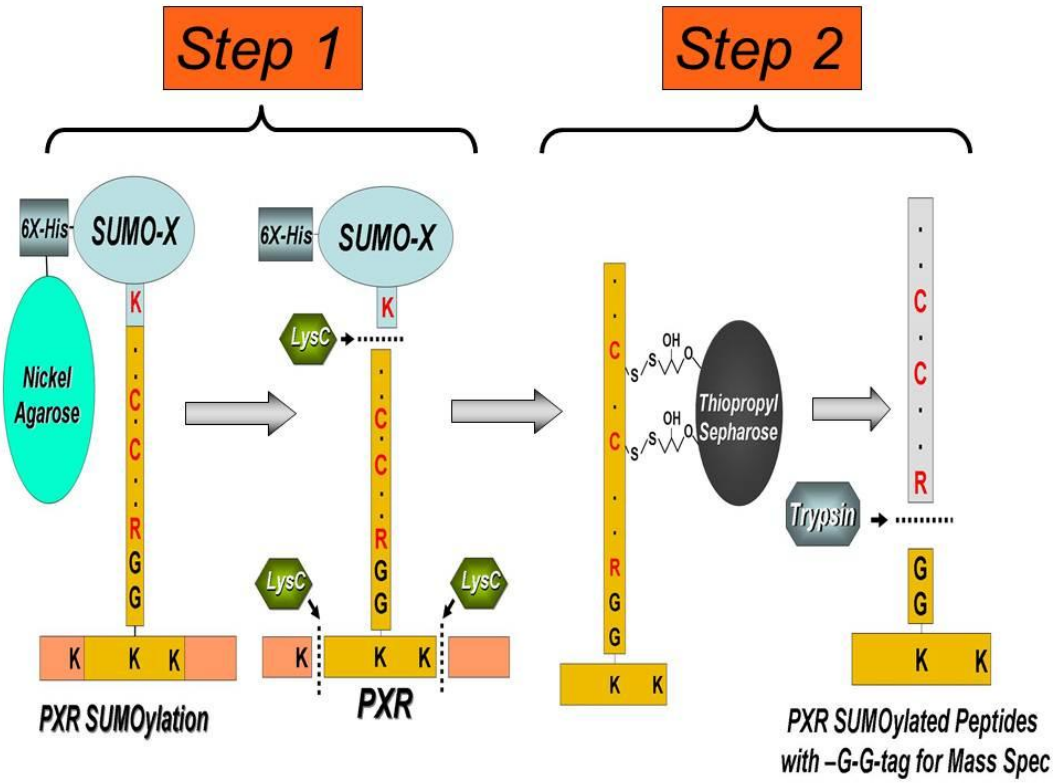


**Figure 3-3. SUMOylation of PXR.** (C) Key altered amino acid residues are colored in RED. Sumo-tagged Lysine residues are protected from digestion with LysC protease. The SUMOylated peptide of interest is indicated with GOLD color. STEP1. The PXR protein is SUMOylated and enriched using Nickel-agarose 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. STEP2. Using thiopropyl sepharose, the SUMOylated cysteine peptides are covalently retained. The target peptides are eluted with trypsin digestion, and the diglycine (GG)-modifed target lysine in PXR is identified as a SUMOylation site using LC-MS/MS.

**Figure 3-3D**

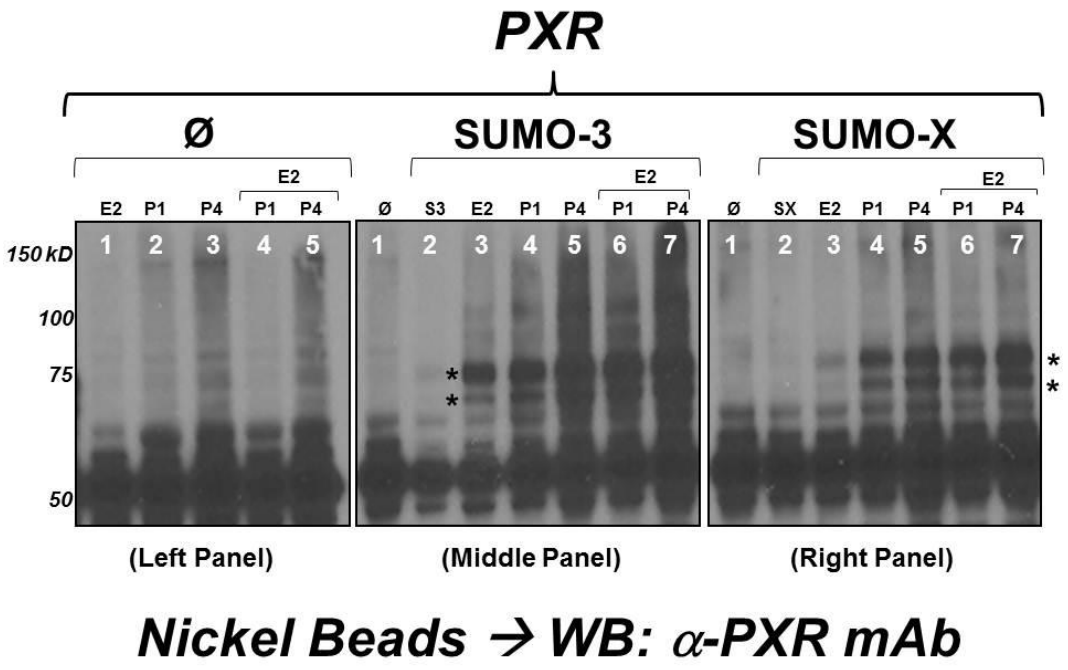


**Figure 3-3. SUMOylation of PXR.** (D) The cDNAs encoding His-SUMO-3, His-SUMOX, Ubc9, PIAS1, or PIAS4 were transfected alone or together as indicated. Isolated PXR protein was detected using a monoclonal antibody that recognizes human PXR.

### *3.3.4 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$\alpha$, 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 3-4A). Acetylation of PXR is readily detected using this experimental approach (Figure 3-4B). 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.

**Figure 3-4A**

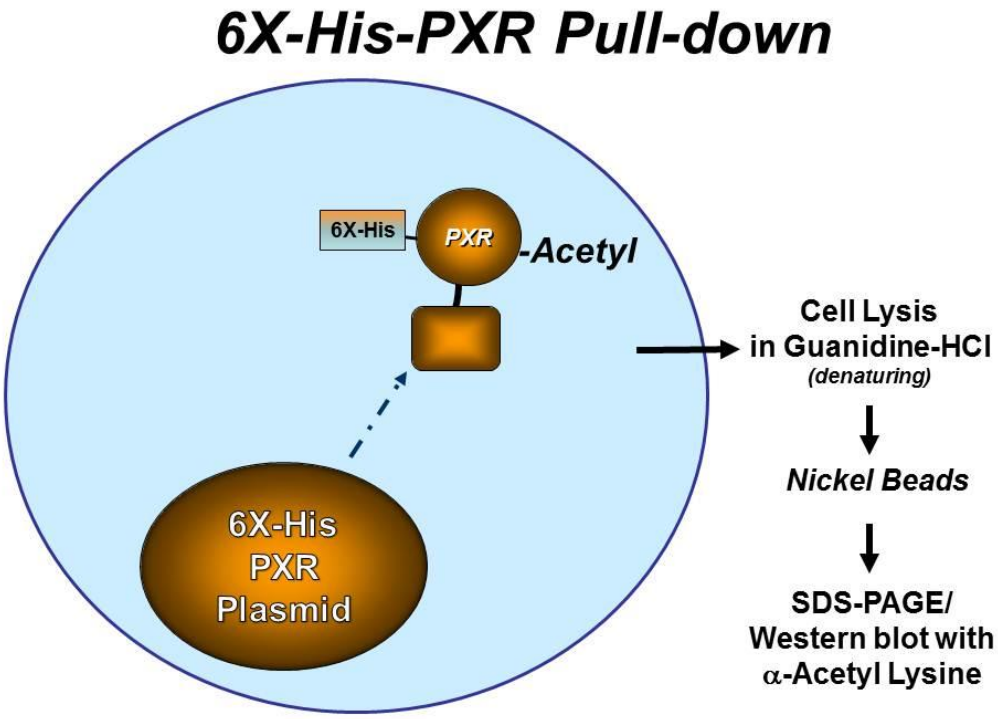


**Figure 3-4. Acetylation of PXR.** (A) Cultured 293T cells are transfected with either a His-tagged human PXR expression construct (pcDNA-His-hPXR) or an empty pcDNA-His vector plasmid as a negative control.

**Figure 3-4B**

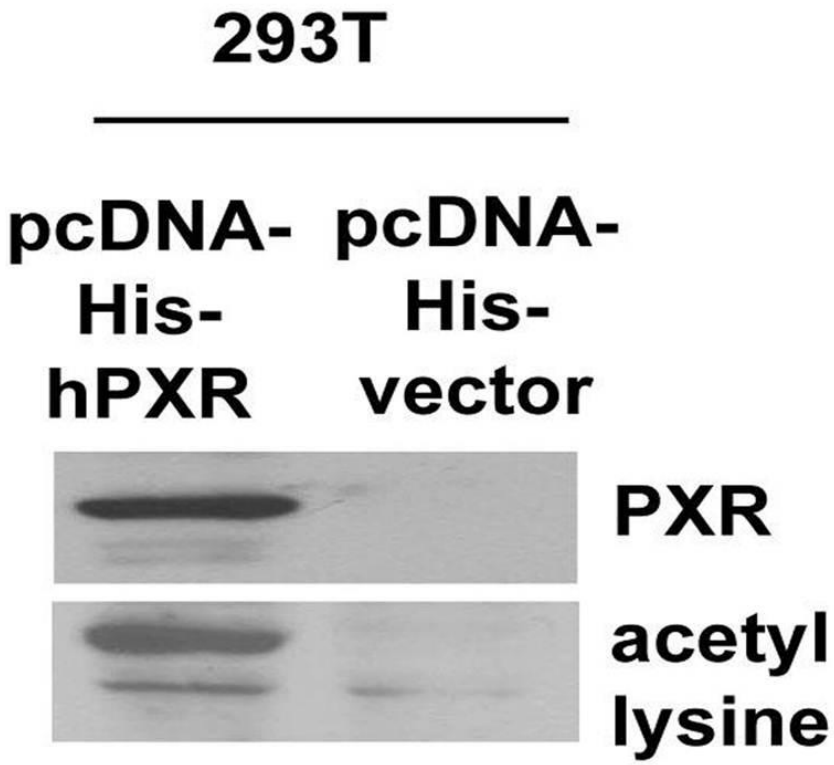


**Figure 3-4. Acetylation of PXR.** (B) Captured proteins from nuclear extracts were subjected to SDS-PAGE and subsequent western blot analysis using an antibody that recognizes either human PXR (top panel) or acetyl-lysine (bottom panel).

### *3.4 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 NFκB 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 where 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.

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# Chapter 4: Regulation of Tissue-specific Carboxylesterase Expression by Pregnane X Receptor and Constitutive Androstane Receptor

### *4.1 Introduction*

Carboxylesterase (CES) enzymes comprise a multi-gene family, and are dominantly involved in hydrolysis activity in liver and small intestine of mammals [1]. These enzymes participate in the biotransformation of a wide range of ester- and amide-containing drugs and prodrugs including angiotensin-converting enzyme inhibitors, anti-tumor drugs, and narcotics such as cocaine and heroin [2-4]. Members of the CES family of enzymes also hydrolyze numerous endogenous compounds including short and long-chain acyl-glycerols, long-chain acyl-carnitine, and long-chain acyl-CoA esters [1].

The mouse *Ces6* gene was first identified in 2004 and encodes a protein of 558 amino acid residues in length [5]. In this same study, the Ces6 gene product was found to hydrolyze selected pyrethroid compounds, a major class of insecticides used worldwide and extensively in the United States. The closest relative of the mouse Ces6 protein in humans is the CES2 enzyme, as these two orthologous proteins are approximately 61% identical and 72% similar when compared at the amino acid level.

Like the human CES2 enzyme, mouse Ces6 is expressed in a liver- and intestine-enriched manner. The CES2 enzyme has come under recent scrutiny because it catalyzes the hydrolysis of several clinically used anti-cancer agents that are administered as pro-drugs. Specifically, CES2 is a high-affinity, high-velocity enzyme with respect to the pro-drug 7-Ethyl-10-[4-(1-piperidino)- 1-piperidino]

carbonyloxy-camptothecin, also called irinotecan, and likely plays a substantial role in irinotecan bioactivation in human liver and intestine at relevant pharmacological concentrations [4]. While much is known regarding the role of carboxylesterase enzymes in biotransformation of prodrugs, little is known regarding the tissue-specific transcriptional regulation of these important drug metabolizing enzymes *in vivo* in tissues such as liver and intestine.

Nuclear receptors comprise a superfamily of ligand-activated transcriptional factors that are involved in diverse physiological, developmental and metabolic processes. The pregnane X receptor (PXR, NR1I2) and constitutive androstane receptor (CAR, NR1I3) are two closely related members of 'xenobiotic-sensing' nuclear receptors among this family. It is now well established that PXR is a key regulator of xenobiotic-inducible *CYP3A* gene expression in liver (reviewed in [6]). PXR also regulates the drug-inducible expression and activity of genes encoding key members of the CYP2B and CYP2C sub-family of enzymes in liver; as well as the drug-inducible expression and activity of several glutathione S-transferase (GST), sulfotransferase (SULT), and UDP-glucuronosyltrasferase (UGT) enzymes in liver [7-10]. PXR-target genes also encode key hepatic drug transporter proteins such as Oatp2, Mdr1/P-glycoprotein, Mrp2, and Mrp3 [11-13]. Recent reports indicate a key role for human PXR in regulating the expression of the *CES2* gene in human hepatocytes and the human hepatoma cell-line, Huh-7, in culture [14]. Other reports suggest a positive role for rat PXR in regulating liver-enriched expression of carboxylesterase enzymes in rodents [10, 15, 16]. Earlier research indicates that

over-expression of constitutively active human PXR increases the expression of mouse genes encoding Ces2 and Ces3 enzymes in mouse liver [17].

The nuclear receptor CAR is also recognized as a 'xenobiotic-sensing' nuclear receptor that is mainly expressed in hepatic tissue. It was originally demonstrated to regulate the phenobarbital (PB)-inducible expression of several genes encoding important members of the CYP2B sub-family of enzymes [18]. CAR has since been shown to regulate the expression and activity of a number of phase I and phase II metabolic enzymes, as well as the expression and activity of numerous important membrane transporter proteins involved in the elimination of endogenous and exogenous substances including bilirubin, steroid hormones, and xenobiotics [19]. Definitive reports linking CAR activation to drug-inducible carboxylesterase gene expression in any species or tissue are currently lacking. However, historical reports indicate that treatment of rodents with PB or PB-like inducers significantly increases carboxyleserase expression and activity in liver tissue [20]. Distinct, yet overlapping functions of PXR and CAR in liver have been described previously [7, 9], and it is well established that these two receptors form the molecular basis of an important class of drug-drug interactions through their actions in liver. While much is known regarding the identity and function of PXR-target genes in liver, less is known about its function in small intestine.

In the present study, we report the identification of several PCN-inducible genes in duodenum of wild type mice using microarray analysis. Among the PCN-inducible genes identified here, expression of the gene encoding the Ces6 protein was further

characterized with respect to its basal and drug-inducible expression in liver and intestine. By exploiting the *Pxr* knockout (PXR-KO) and *Car* knockout (CAR-KO) mouse models, we reveal that in both duodenum and liver tissues, the drug-inducible expression of *Ces6* is regulated by both PXR and CAR. Our data conclusively show that *Ces6* is a shared PXR- and CAR-target gene in mice. Interestingly, in small intestine, despite significant expression of the CAR nuclear receptor, expression of the *Ces6* gene is exclusively regulated by *i.p.* treatment with the prototypical CAR agonist 1,4-bis[2-(3,5-dichloropyridyloxy)] benzene (TCPOBOP) and not by *i.p.* treatment of mice with PB. These data suggest that there may be significant differences in the bioavailability of PB and TCPOBOP in intestine following treatment with these two CAR activators that can produce variable results. Alternatively, differences in the mode of CAR activation, either phosphorylation-dependent in the case of PB or direct ligand-mediated activation in the case of TCPOBOP, can likely produce tissue-specific differences when using these two compounds to activate the CAR nuclear receptor in intestine and liver tissue *in vivo*. In any case, these data reveal that liver- and intestine-enriched carboxylesterase expression and activity is likely modulated in humans on combination therapy in a clinically significant manner. This is of particular importance as numerous drug development programs seek to take advantage of intestine- and liver-enriched carboxylesterase enzymes as convenient targets for delivery of increasing numbers of pro-drugs.

### *4.2 Materials and Methods*

***Animal Care.*** All rodents were maintained on standard laboratory chow and allowed food and water *ad libitum*. All mice were treated once a day i.p. with either vehicle (corn oil, saline), pregnenalone 16-carbonitrile (PCN) (100 mg/kg in corn oil), TCPOBOP (3 mg/kg in corn oil), or phenobarbital (PB) (100 mg/kg in saline) for 4 days. The studies reported here have been carried out in accordance with the Declaration of Helsinki and/or with the *Guide for the Care and Use of Laboratory Animals* as adopted and promulgated by the National Institutes of Health.

***Drugs and Chemicals.*** Unless otherwise stated, all chemical compounds were purchased from Sigma-Aldrich (St. Louis, Mo). Antibody against CES6 was purchased from Abcam Inc. (Cambridge, Ma). The anti-Cyp3a23 antibody that cross reacts with mouse Cyp3a11 protein was used to probe immunoblots (Chemicon). The anti-Cyp2b10 antibody was obtained from Millipore (Temecula, Ca)

***Microarray Analysis.*** Total RNA was isolated from duodenums of wild-type mice using TRIzol reagent (Invitrogen) according to the manufacturer's instructions. The quality and quantity of the total RNA samples were examined with both UV spectrophotometry using a Nanodrop (ND-1000) and Bioanalyzer 2100. For the subsequent micorarray study, Affymetrix Mouse Genome GeneChip 430 2.0 oligonucleotide arrays were employed that cover over 39,000 transcripts from the mouse genome. To carry out the GeneChip analysis, established standard protocols at the University of Kansas Genomics Facility were performed on cRNA target preparation, array hybridization, washing, staining and image scanning. After they

were generated from the Affymetrix GeneChip Operating Software (GCOS.v1.2), the microarray data was first subjected to quality assessment. All GeneChip data passed the quality control (QC) step since data of all chips met established Affymetrix GeneChip QC criteria including low background, low noise, positive detection of QC probe sets such as bioB, percentage of called present in the normal range (40-60%), similar scaling factors across chips, and 3'/5' ratio. Due to its known response to PCN, *Cyp3a11* was also used as a positive control in this study.

***Microarray Data Analysis.*** To facilitate direct comparison of gene expression data between the PCN- and vehicle-treated samples, the GeneChip data were first subjected to preprocessing including background correction, probe summarization and normalization using the Affymetrix MAS5 algorithm. All chips were scaled to a target signal of 500. Prior to identification of differentially expressed genes, genes that were called 'Absent' by the MAS5 algorithm were filtered out. A volcano-plot based approach was subsequently used to identify PCN-inducible gene expression. The expression value of a PCN-inducible gene had to pass two criteria, (1) the fold change between the compound treatment and the negative control had to be $\geq 1.5$, and (2) the P value from parametric test using all available error estimates had to be $\leq 0.05$.

***Real-time Quantitative PCR.*** After DNase I treatment, 1 μg of RNA was reverse transcribed using random primers following the manufacturer's instruction (Promega). Equal amounts of cDNA were used in real-time quantitative PCR reactions. Reactions included 200 nM fluorogenic probe and 300 nM primers specific for each gene. The fluorogenic probe and primer sets were designed using the Primer3

program (http://frodo.wi.mit.edu/). The fluorogenic probes were synthesized by BioSearch Technologies (Novato, CA). The sequences (5' to 3') for the primers and probes are as follows: *Cyp3a11*, forward primer (CAAGGAGATGTTCCCTGTCA), fluorogenic probe (FAM-AGAAGGC AAAGAAAGGCAAGCCTG-BHQ1), reverse primer (CCACGTTCACTCC AAATGAT); *Cyp2b10*, forward primer (GACTTTGGGATGGGAAAGAG), fluorogenic probe (FAM-TAGTGGAGGAACTGCGGAAATCCC-BHQ1), reverse primer (CCAAACACAATGGAGCAGAT). For the Ces6 and 18S genes, 1× SYBR Green (BioWhittaker Molecular Applications) was included in the reaction instead of the fluorogenic probe. The sequences (5' to 3') for the Ces6 and 18S are as follows: *Ces6*, forward primer (GTGTGAGAGATGGGACCTCA), reverse primer (TCATTCATGGAAGCTGATCC); 18S, forward primer (AGTCCCTGCCCTTTGTACACA), reverse primer (CGATCCGAGGGC CTCACTA). Cycling conditions were 95 $^{0}$C for 2 min followed by 45 cycles of 95 $^{0}$C for 15 s, 60 $^{0}$C for 15 s, and 68 $^{0}$C for 15 s using the Cepheid Smart Cycler system (Sunnyvale, CA). The fold induction was calculated as described previously [13].

***Northern Blot Analysis.*** Total RNA was isolated as described in Real-time quantitative PCR, and 20 μg total RNA per lane were resolved on 3.7% formaldehyde/1% agarose gel in MOPS buffer for northern blot analysis. Blots were hybridized with $^{32}$P-labeled cDNA corresponding to the sequences for mouse *Ces6*, *Cyp3a11*, and *Cyp2b10*, and 18S ribosomal RNA as described previously [21].

***Western Blot Analysis.*** Approximatedly 250 mg of liver tissue was homogenized using a Dounce Teflon homogenizer in 1 ml of homogenization buffer (50 mM Tris-HCl, pH 7.4 at $4^0$C containing 150 mM KCl and 2 mM EDTA). The homogenate was subjected to centrifugation at 500 x g for 15 min at 4 $^0$C to remove cell debris and nuclei. The supernatant fraction was subjected to centrifugation at 12,000 x g for 15 min at $4^0$C. Microsomes were prepared by ultracentrifugation (50,000 x g for 60 min at $4^0$C) of the postmitochondrial supernatant fraction. The first microsomal pellet was resuspended in wash buffer (10 mM EDTA containing 150 mM KCl), then re-isolated by ultracentrifugation (50,000 x g for 60 min at $4^0$C). The washed microsomes were suspended in a small volume of 250 mM sucrose. Liver microsomal protein (20 μg/lane) were resolved on 10% SDS-PAGE gels and transferred to PVDF microporous membranes (Millipore) that were probed with anti-Ces6, anti-Cyp3A23, and anti-Cyp2b10 antibodies. Immunodetection was performed using the ECL kit (Amersham) according to the protocol provided by the manufacturer. Quantitative densotometric analyses of western blot images were achieved using the digital Kodak EDAS 290 image acquisition system together with the 1D image analysis software package.

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

### *4.3 Results*

***The Basal Expression Profiles of Mouse Cyp3a11, Pxr, and Car in Liver, Duodenum, Jejunum, and Ileum.*** Liver and small intestine are two major organs that play an important and primary role in regulating the metabolism, transport, excretion, and efflux of xenobiotic compounds. The relative expression levels of mouse genes encoding *Cyp3a11*, *Pxr*, and *Car* in three individual wild type mice were determined using total RNA isolated from liver, duodenum, jejunum and ileum (Figure 4-1). The expression of the *Cyp3a11* gene was highest in mouse liver, followed by a significant reduction of approximately 60% in duodenum. The *Cyp3a11* transcript in jejunum and ileum was virtually undetectable. The expression level of *Pxr* was also determined. The *Pxr* mRNA was expressed at the highest levels in liver, with lower but easily detectable levels observed in duodenum, jejunum, and ileum. The *Pxr* mRNA was approximately 60% of that observed in liver when examined in duodenum, jejunum, and ileum, respectively. The expression level of *Car* was then determined. The *Car* gene was expressed at the highest level in liver, with slightly lower but easily detectable expression levels observed in duodenum, jejunum, and ileum.

***Detection of PCN-inducible Cyp3a11 Gene Expression in Duodenum.*** Prior to performing microarray analysis, we sought to determine whether the known PXR-target gene, *Cyp3a11*, was induced in small intestine following administration of PCN, a well-known rodent PXR activator. Wild type mice (n=3) were treated for four days using an *i.p.* injection of either vehicle (corn oil) or PCN (100 mg/kg). On the morning of day 5, tissues were harvested and total RNA was isolated from the entire

**Figure 4-1**

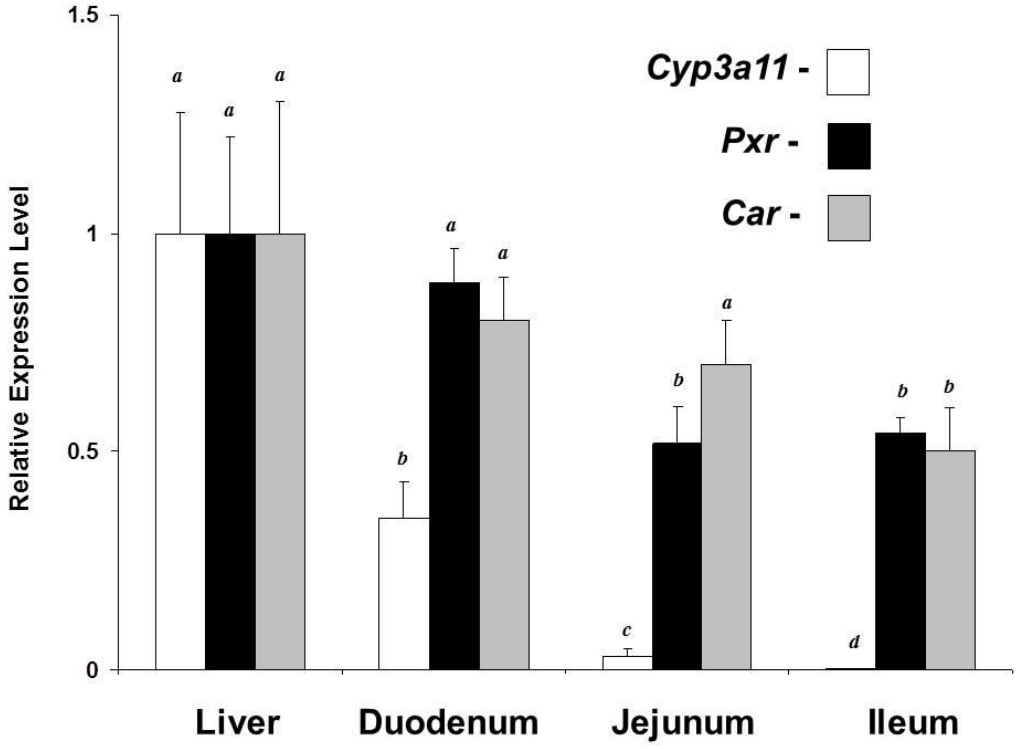


**Figure 4-1. The basal expression of *Cyp3a11*, *Pxr*, and *Car* gene expression levels in liver, duodenum, jejunum, and ileum.** Total RNA was isolated from liver, duodenum, jejunum, and ileum of 6-week old wild type mice. Real-time quantitative PCR analysis was performed in order to measure the relative abundance of the transcripts using reverse transcribed cDNA from all tissues examined. All data are normalized to 18S levels and are expressed relative to that observed in liver and represent average values ± SEM (n = 3). Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

small intestine and resolved on an agarose gel for subsequent northern blotting using a cDNA probe to detect *Cyp3a11* expression levels. This analysis revealed that *Cyp3a11* gene expression was induced in small intestine by treatment with PCN (*data not shown*). These data indicate that PXR-mediated target gene activation is completely intact in the small intestine of wild type mice. Induction of *Cyp3a11* gene expression was absent from duodenal tissue isolated from PXR-KO mice (data not shown). Taken together with the data obtained in figure 4-1, we reasoned that treatment of wild type mice with PCN should produce robust induction of PXR-target genes in the duodenum and that microarray analysis should detect PXR-mediated gene activation.

***Detection of PCN-responsive Genes in Mouse Duodenum by Microarray.*** In order to identify novel PXR-target genes in mouse duodenum, we performed microarray analyses using GeneChip Mouse Genome 430 2.0 oligonucleotide arrays from Affymetrix, which interrogate over 39,000 transcripts encompassing the entire mouse genome. As expected, the expression of the prototypical PXR-target gene, *Cyp3a11*, was increased approximately 1.8-fold following treatment with the known PXR activator, PCN, in duodenum. Several additional CYP genes were increased by treatment with PCN including *Cyp2C55*, *Cyp2C29*, and *Cyp3a25*, by approximately 3.9-fold, 3.3-fold, and 2.0-fold, respectively. Seven genes encoding distinct members of the glutathione S-transferase family of enzymes were up-regulated following treatment with PCN. Three different glutathione S-transferase mu isoenzymes were increased following treatment with PCN including the Gstm1, Gstm3, and Gstm6

isoforms by 5.3-fold, 2.6-fold, and 1.9-fold, respectively. The glutathione S-transferase Gsta2 and Gsta4 isoforms were increased following treatment with PCN by 2.2-fold and 1.9-fold, respectively. The microsomal glutathione S-transferase Mgst1 and Mgst2 enzymes were increased 1.6-fold and 2.0-fold, respectively. The gene encoding microsomal epoxide hydrolase was increased approximately 7.0-fold following treatment with PCN. Table 4-1 shows a complete listing of selected genes identified following this analysis. The gene that was induced to the highest absolute level as judged by the overall strength of the signal detected from the PCN-treated sample following *Cyp3a11* and *Gsta2* was *Ces6*. Therefore, we chose to further characterize the potential role of PXR and CAR in modulating the drug-inducible expression of this gene in both liver and intestine.

***Regulation of Ces6 mRNA by PCN in Mouse Duodenum is PXR-dependent.*** To determine whether *Ces6* represents a *bona fide* PXR-target gene in duodenum, we performed both northern blot and real-time quantitative PCR (rt-QPCR) analysis using wild type and PXR-KO mice. Northern blot analysis using total RNA isolated from the duodenum of three individual animals was performed to determine the relative expression levels of both *Cyp3a11* and *Ces6* (Figure 4-2A). Treatment of wild type mice with PCN produced marked increases in *Ces6* and *Cyp3a11* mRNA levels, however, PCN-inducible increases in the expression of both the *Ces6* and *Cyp3a11* mRNAs were totally absent when the analysis was performed using total RNA isolated from the duodenum of individual PXR-KO mice. The results obtained using northern blot analysis were quantified using rt-QPCR (Figure 4-2B). The *Ces6* (*left panel*) and

**Table 4-1. Genes Up-regulated by PCN Treatment in Duodenum**

| Gene | Fold Induction | Description |
|---|---|---|
| Ephx1 | 7.0 | Epoxide hydrolase 1, microsomal |
| Gstm1 | 5.3 | Glutathione S-transferase, mu 1 |
| Gstm3 | 2.6 | Glutathione S-transferase, mu 3 |
| Gstm6 | 1.9 | Glutathione S-transferase, mu 6 |
| Cyp2c55 | 3.9 | Cytochrome P450, family 2, subfamily c, polypeptide 55 |
| Cyp2c29 | 3.3 | Cytochrome P450, family 2, subfamily c, polypeptide 29 |
| Cyp3a25 | 2.0 | Cytochrome P450, family 3, subfamily a, polypeptide 25 |
| Cyp3a11 | 1.8 | Cytochrome P450, family 3, subfamily a, polypeptide 11 |
| Ces6 | 3.2 | Carboxylesterase 6 |
| Akr1b7 | 3.0 | Aldo-keto reductase family 1, member B7 |
| Hsd17b11 | 2.4 | Hydroxysteroid 17-beta dehydrogenase 9 |
| Gsta4 | 2.2 | Glutathione S-transferase, alpha 4 |
| Gsta2 | 1.9 | Glutathione S-transferase, alpha 2 (Yc2) |
| Mgst2 | 2.0 | Microsomal glutathione S-transferase 2 |
| Mgst1 | 1.6 | Microsomal glutathione S-transferase 1 |
| Abca1 | 2.0 | ATP-binding cassette, sub-family A (ABC1), member 1 |
| Aldh1a7 | 1.9 | Aldehyde dehydrogenase family 1, subfamily A1 |
| Pdk4 | 1.8 | Pyruvate dehydrogenase kinase 4 |
| Sgk | 1.7 | Serum/glucocorticoid regulated kinase |
| Hpgd | 1.7 | Hydroxyprostaglandin dehydrogenase 15 (NAD) |
| Dbi | 1.6 | Diazepam binding inhibitor |

**Figure 4-2A**

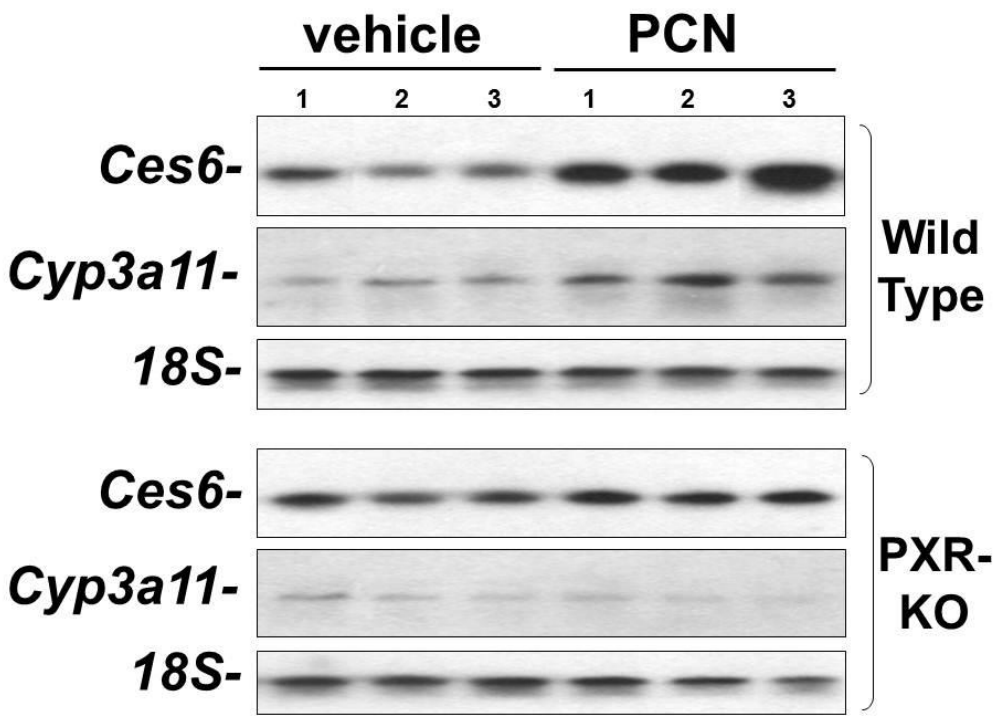


**Figure 4-2. Induction of *Ces6* and *Cyp3a11* gene expression by PCN treatment in mouse duodenum is PXR-dependent.** Total RNA was isolated from the duodenum of wild type and PXR knockout mice (*n*=3) treated *i.p.* with vehicle (corn oil) or PCN (100 mg/kg) for 4 days. (A) Northern blot analysis was performed using 20 μg total RNA/lane and the blots were probed sequentially with $^{32}$P-labeled cDNA fragments encoding *Ces6*, *Cyp3a11*, and 18S ribosomal RNA. Each lane represents an individual animal.

**Figure 4-2B**

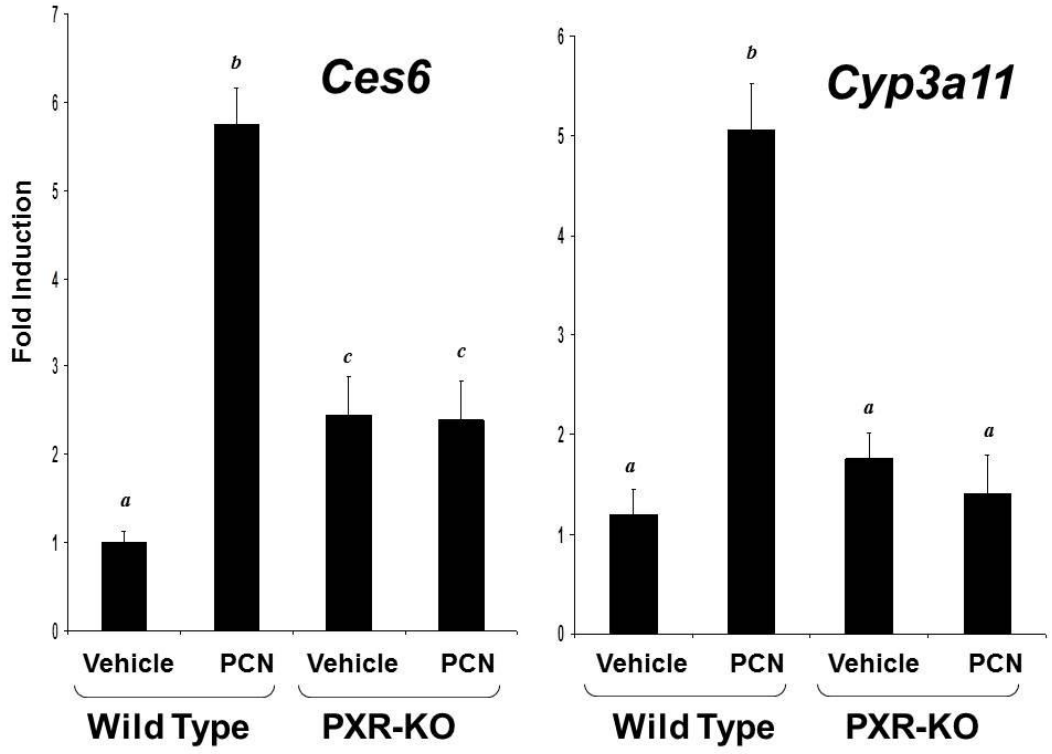


**Figure 4-2. Induction of *Ces6* and *Cyp3a11* gene expression by PCN treatment in mouse duodenum is PXR-dependent.** Total RNA was isolated from the duodenum of wild type and PXR knockout mice ($n$=3) treated *i.p.* with vehicle (corn oil) or PCN (100 mg/kg) for 4 days. (B) Real-time quantitative PCR analyses were performed to determine the expression levels of *Ces6* and *Cyp3a11*. All data are normalized to 18S levels and represent the average values ± SEM ($n$=5) and are expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p$<0.05).

*Cyp3a11* (*right panel*) mRNAs exhibited a significant increase in their expression level, respectively, with each gene exhibiting an approximate increase of 5-fold in wild type mice. In contrast, no significant increases in expression were detected when total RNA was analyzed from the duodenum of PXR-KO mice.

***Regulation of Drug-inducible Ces6 is PXR- and CAR-dependent.*** A number of studies have shown that PCN treatment induces *Cyp3a11* gene expression in both mouse liver and intestine [22-24]. Other research has shown that PXR and CAR share distinct but overlapping target genes in liver [9, 25-27]. We next investigated whether induction of *Ces6* gene expression in liver and intestine by treatment with PCN, TCPOBOP, or PB is dependent upon PXR or CAR. We therefore treated wild type, PXR-KO, and CAR-KO mice with vehicle, PCN, TCPOBOP, or PB and isolated total RNA from liver and duodenum and microsomes from liver for subsequent analysis of gene expression and protein levels, respectively. We first analyzed *Ces6* and *Cyp3a11* gene expression levels using northern blot analysis (Figure 4-3A). As expected, treatment of wild type mice with PCN produced increased levels of *Cyp3a11* gene expression in liver in a PXR-dependent manner. Treatment with PCN also produced increased *Ces6* mRNA levels in liver in a PXR-dependent manner. Subsequent quantitative analysis using rt-QPCR analysis revealed that statistically significant induction of both *Ces6* (*left panel*) and *Cyp3a11* (*right panel*) gene expression by PCN treatment in mouse liver is PXR-dependent (Figure 4-3B).

We next examined the relative expression levels of microsomal Ces6 and Cyp3a11 protein in mouse liver. Hepatic microsomes were isolated and resolved on an

**Figure 4-3A**

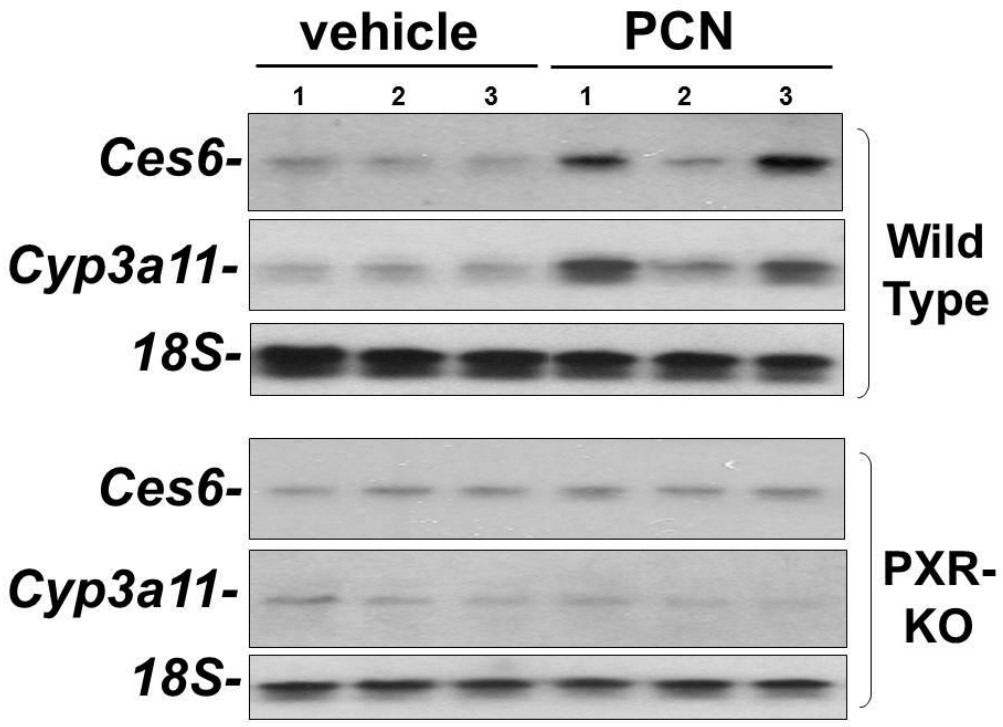


**Figure 4-3. Induction of *Ces6* and *Cyp3a11* gene expression by PCN treatment in mouse liver is PXR-dependent**. Total RNA was isolated from the liver of wild type and PXR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or PCN (100 mg/kg) for 4 days. (A) Northern blot analysis was performed using 20 μg total RNA/lane and the blots were probed sequentially with $^{32}$P-labeled cDNA fragments encoding *Ces6*, *Cyp3a11* and 18S ribosomal RNA. Each lane represents an individual animal.

**Figure 4-3B**

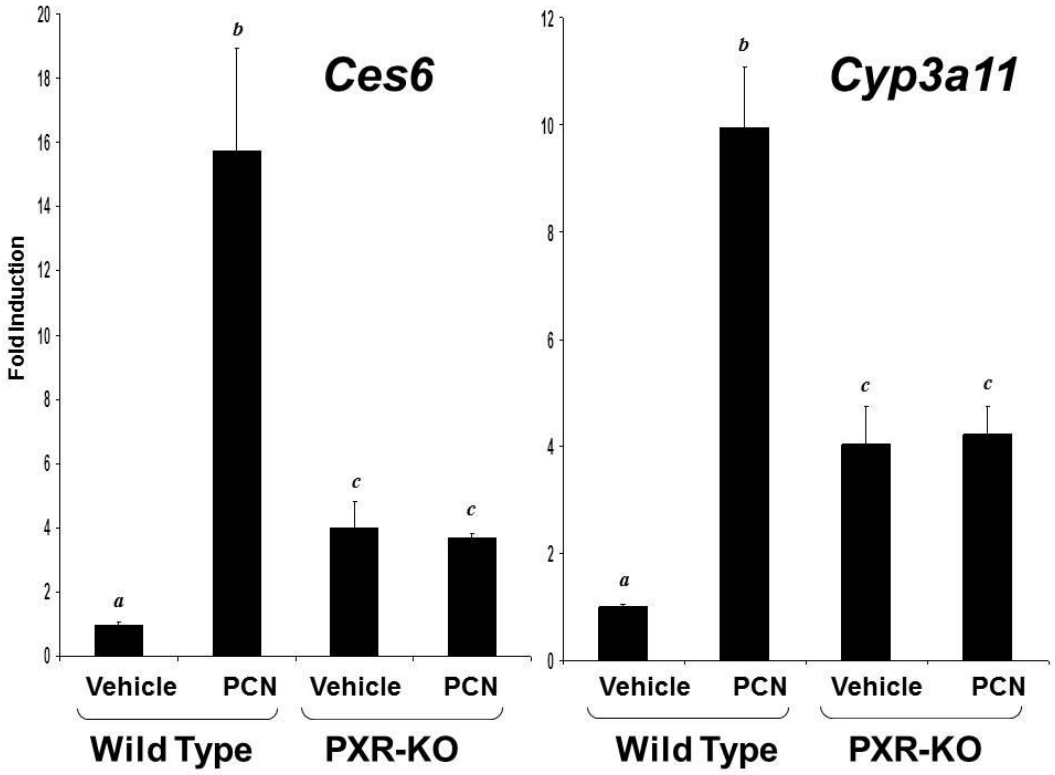


**Figure 4-3. Induction of *Ces6* and *Cyp3a11* gene expression by PCN treatment in mouse liver is PXR-dependent**. Total RNA was isolated from the liver of wild type and PXR knockout mice ($n$=3) treated *i.p.* with corn oil (vehicle) or PCN (100 mg/kg) for 4 days. (B) Real-time quantitative PCR analyses were performed to determine the expression of *Cyp3a11* and *Ces6*. All data are normalized to 18S levels and represent the average values ± SEM ($n$=5) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p$<0.05).

SDS-PAGE gel for subsequent western blot analysis. Expression of both the Ces6 and Cyp3a11 proteins were induced by PCN treatment in a PXR-dependent manner in hepatic microsomes (Figure 4-4A). Quantitative analyses of these data indicate that both Ces6 (*left panel*) and Cyp3a11 (*right panel*) protein levels are significantly induced in liver in a PXR-dependent manner (Figure 4-4B). Deletion of the PXR protein *in vivo* significantly increased the basal expression of both Ces6 and Cyp3a11 protein levels.

We next sought to determine whether expression of the *Ces6* gene in liver is regulated by the nuclear receptor CAR. Mice were treated for four days *i.p.* with vehicle, TCPOBOP, or PB. Liver and duodenum were removed and total RNA and protein was isolated on the morning of day 5. Gene expression levels were qualitatively and quantitatively determined using northern blot and rt-QPCR analyses, respectively. In liver, the expression of genes encoding *Cyp2b10* and *Ces6* were increased following treatment with TCPOBOP in a CAR-dependent manner in liver (Figure 4-5A). Quantitative analysis using rt-QPCR confirmed that both *Ces6* and *Cyp2b10* gene expression levels were significantly induced by treatment with TCPOBOP in liver and duodenum in a CAR-dependent manner (Figure 4-5B).

We next examined Ces6 and Cyp2B10 protein levels in mouse hepatic microsomes qualitatively and quantitatively using western blot analysis and photo-densitometry, respectively. These analyses revealed that treatment of mice with TCPOBOP produced robust increases in hepatic microsomal Ces6 and Cyp2b10 proteins in a

**Figure 4-4A**

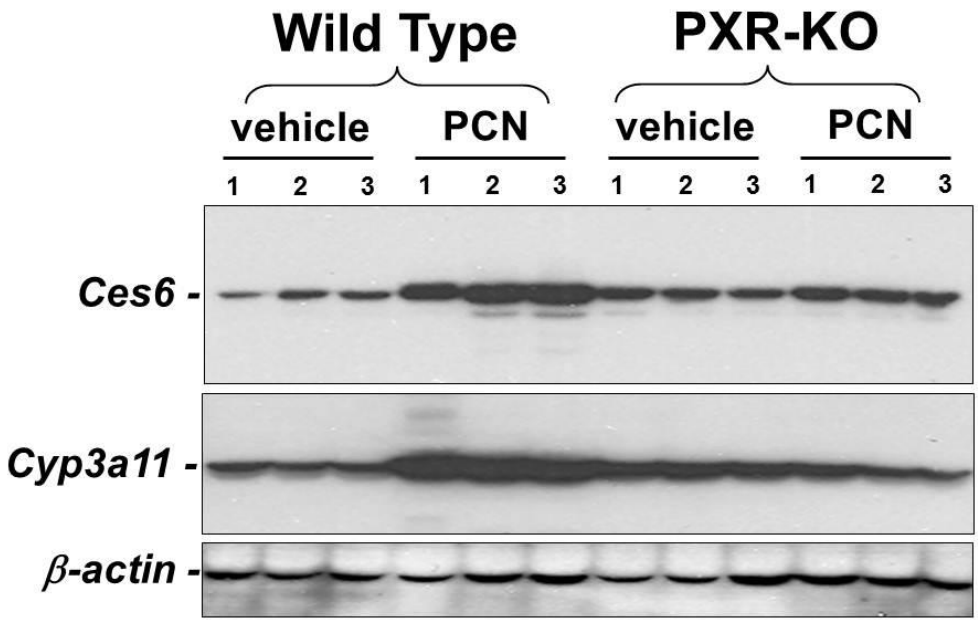


**Figure 4-4. Expression of Ces6 and Cyp3a11 protein is induced by PCN in a PXR-dependent manner in mouse liver.** Hepatic microsomes were from the liver of wild type and PXR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or PCN (100 mg/kg) for 4 days. (A) Western blot analysis was performed to determine the expression levels of Ces6, Cyp3a11 and β-actin protein, respectively. Each lane represents an individual animal.

**Figure 4-4B**

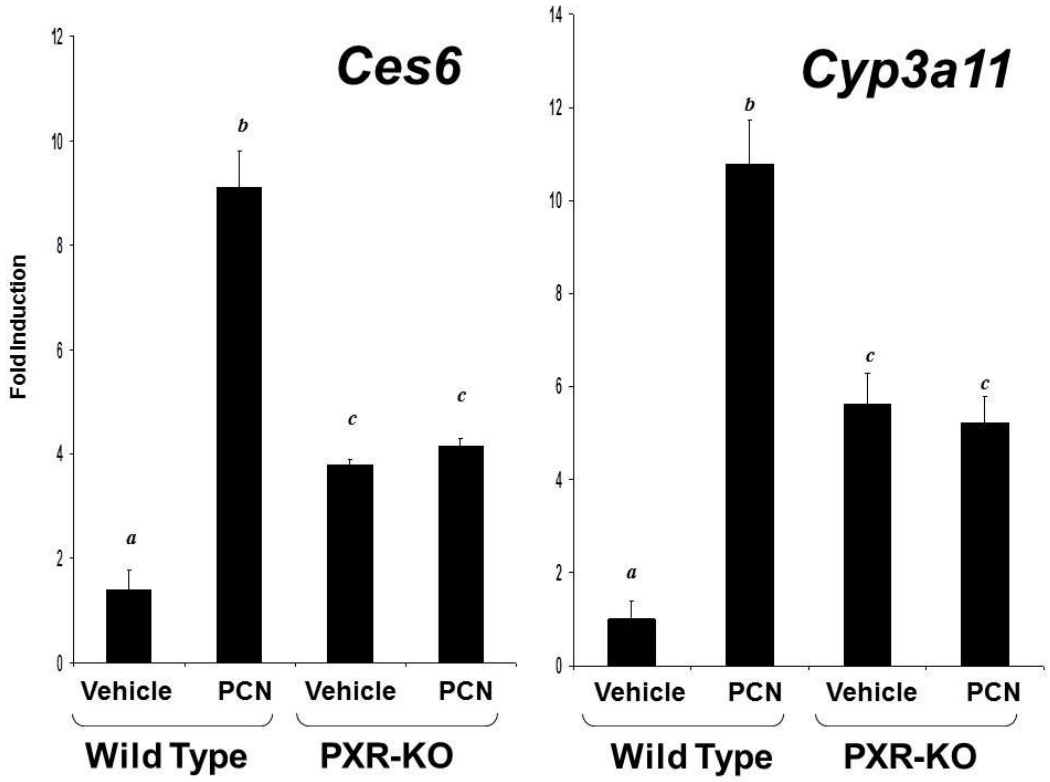


**Figure 4-4. Expression of Ces6 and Cyp3a11 protein is induced by PCN in a PXR-dependent manner in mouse liver.** Hepatic microsomes were from the liver of wild type and PXR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or PCN (100 mg/kg) for 4 days. (B) The results from (A) were quantified using scanning densitometry. All data are normalized to β-actin levels and represent the average values ± SEM (*n=3*) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

**Figure 4-5A**

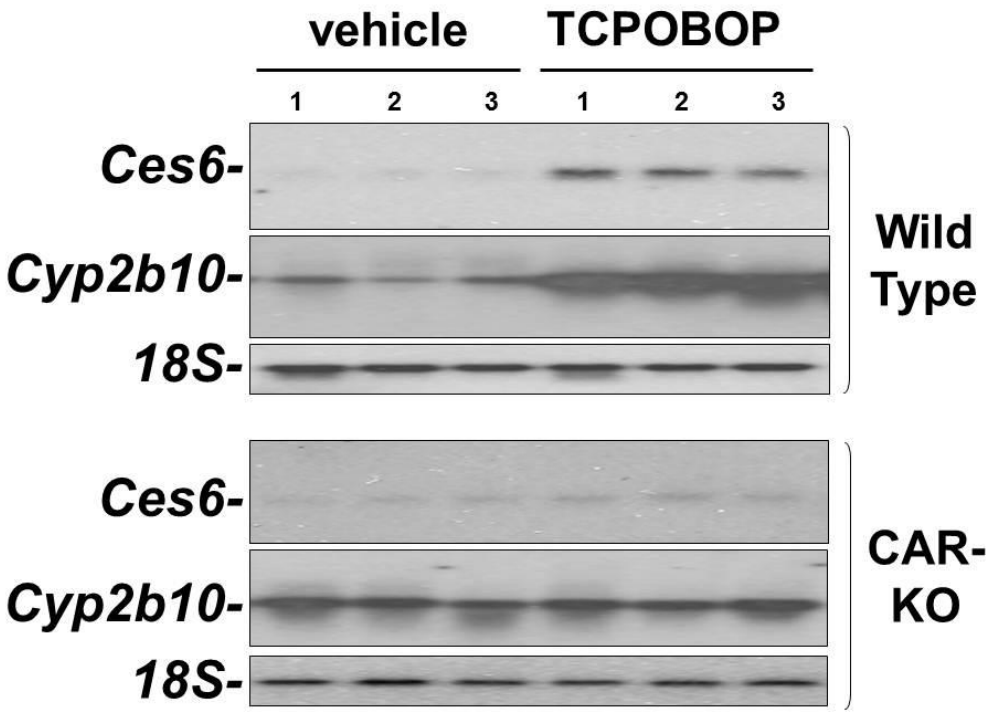


**Figure 4-5. Induction of *Ces6* and *Cyp2b10* gene expression by TCPOBOP treatment in mouse liver is CAR-dependent**. Total RNA was isolated from the liver of wild type and CAR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or TCPOBOP (3 mg/kg) for 4 days. (A) Northern blot analysis was performed using 20 μg total RNA/lane and the blots were probed sequentially with $^{32}$P-labeled cDNA fragments encoding *Ces6*, *Cyp2b10* and 18S ribosomal RNA. Each lane represents an individual animal.

**Figure 4-5B**

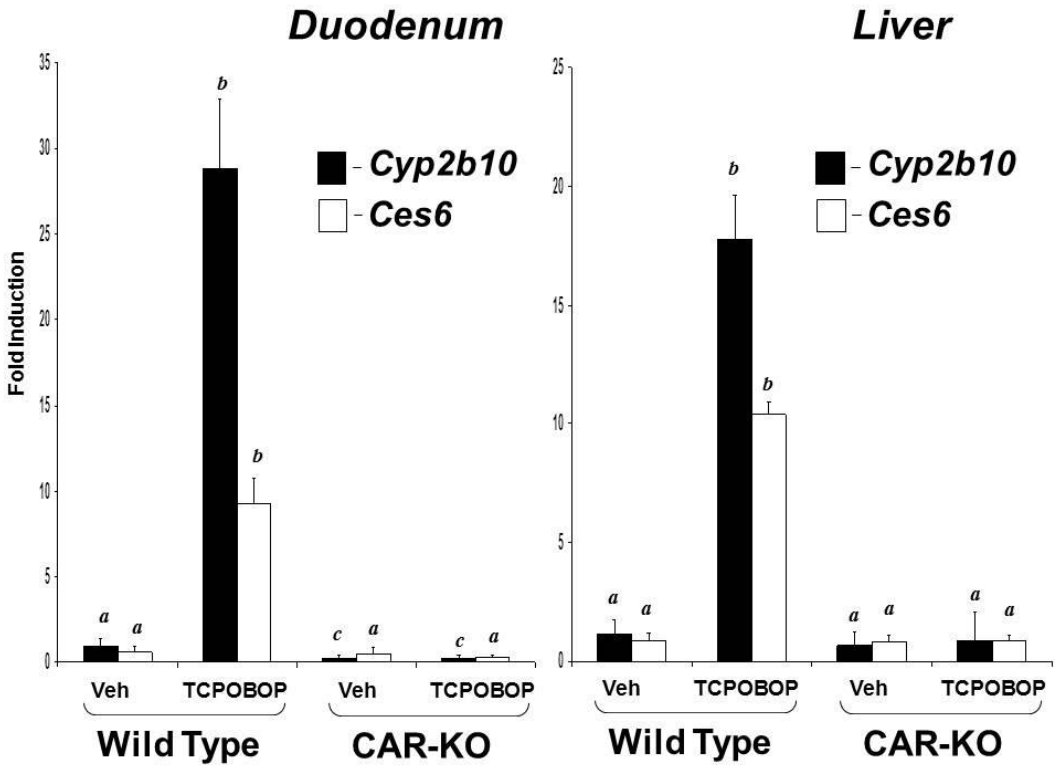


**Figure 4-5. Induction of *Ces6* and *Cyp2b10* gene expression by TCPOBOP treatment in mouse liver is CAR-dependent**. Total RNA was isolated from the liver of wild type and CAR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or TCPOBOP (3 mg/kg) for 4 days. (B) Real-time quantitative PCR analyses were performed to determine the expression levels of *Ces6* and *Cyp2b10* in duodenum and liver tissues. All data are normalized to 18S levels and represent the average values ± SEM (*n=5*) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

CAR-dependent manner in liver microsomal fractions (Figure 4-6A). Densotimetric analysis showed that the increased levels of Ces6 (*left panel*) and Cyp2b10 (*right panel*) protein observed in wild type mice treated with TCPOBOP was statistically significant (Figure 4-6B). Similar results in liver were obtained when PB treatment was used to activate the CAR nuclear receptor protein *in vivo*. Qualitative analysis using northern blotting revealed that PB treatment produced robust increases in the expression levels of the genes encoding *Ces6* and *Cyp2b10* in a CAR-dependent manner in liver (Figure 4-7A). Quantitative analysis using rt-QPCR revealed that treatment with PB produced significant increases in the *Ces6* and *Cyp2b10* genes in liver (Figure 4-7B). Examination of Ces6 and Cyp2B10 protein levels in mouse hepatic microsomes was accomplished qualitatively and quantitatively using western blot analysis and photo-densitometry, respectively. These analyses revealed that treatment of mice with PB produced robust increases in hepatic microsomal Ces6 and Cyp2b10 proteins in a CAR-dependent manner in liver microsomal fractions (Figure 4-8A). Densotimetric analysis showed that the increased levels of Ces6 (*left panel*) and Cyp2b10 (*right panel*) protein observed in wild type mice treated with TCPOBOP was statistically significant (Figure 4-8B). Interestingly, no increases in the basal levels of either Ces6 or Cyp2b10 proteins were observed on CAR-KO mice. This is in stark contrast to that observed in the PXR-KO mice. This observation likely indicates that the CAR protein does not play an active repressive role in a non-stimulated state in liver. Treatment of all genotypes of mice examined here with PB did not produce any significant changes in expression levels of genes encoding *Ces6*, *Cyp3a11*, or *Cyp2b10*

**Figure 4-6A**

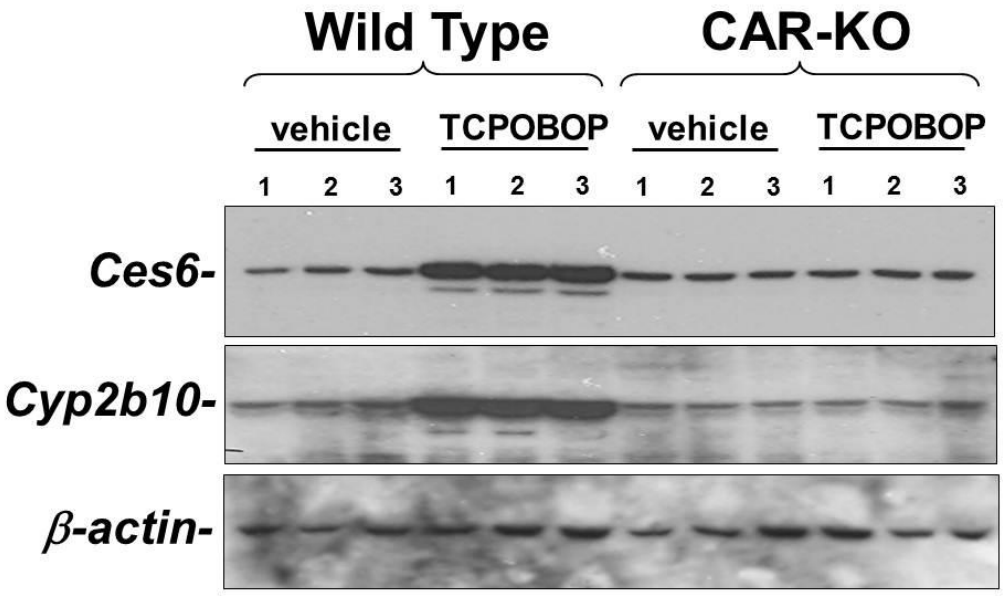


**Figure 4-6. Expression of Ces6 and Cyp2b10 protein is induced by TCPOBOP in a CAR-dependent manner in mouse liver.** Hepatic microsomes were from the liver of wild type and CAR knockout mice (*n*=3) treated *i.p.* with corn oil (vehicle) or TCPOBOP (3 mg/kg) for 4 days. (A) Western blot analysis was performed to determine the expression levels of Ces6, Cyp2b10 and β-actin protein, respectively. Each lane represents an individual animal.

**Figure 4-6B**

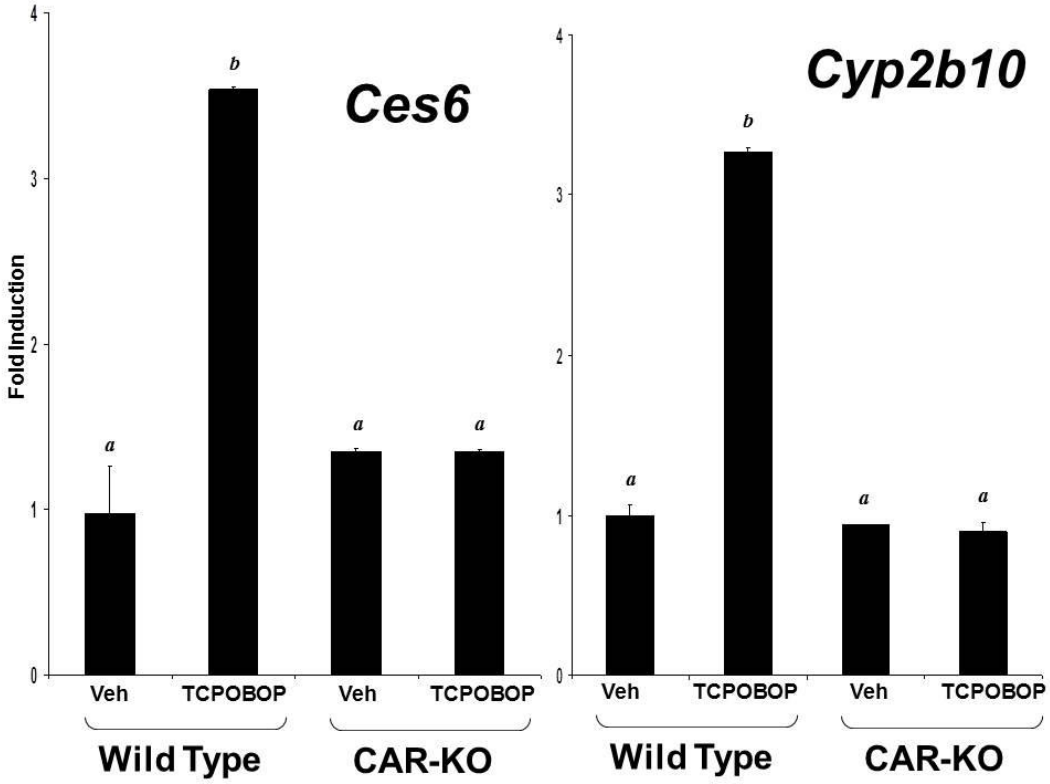


**Figure 4-6. Expression of Ces6 and Cyp2b10 protein is induced by TCPOBOP in a CAR-dependent manner in mouse liver.** Hepatic microsomes were from the liver of wild type and CAR knockout mice ($n$=3) treated *i.p.* with corn oil (vehicle) or TCPOBOP (3 mg/kg) for 4 days. (B) The results from (A) were quantified using scanning densitometry. All data are normalized to β-actin levels and represent the average values ± SEM ($n=3$) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

**Figure 4-7A**

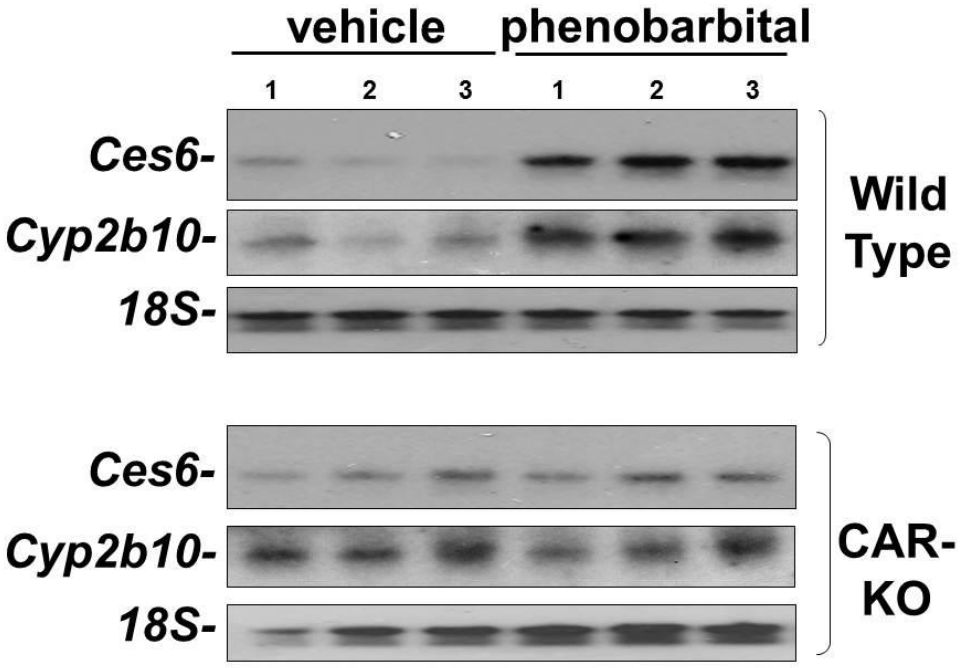


**Figure 4-7. Induction of *Ces6* and *Cyp2b10* gene expression by phenobarbital treatment in mouse liver is CAR-dependent.** Total RNA was isolated from the liver of wild type and CAR knockout mice (*n*=3) treated *i.p.* with saline (vehicle) or PB (100 mg/kg) for 4 days. (A) Northern blot analysis was performed using 20 μg total RNA/lane and the blots were probed sequentially with $^{32}$P-labeled cDNA fragments encoding *Ces6*, *Cyp2b10* and 18S ribosomal RNA. Each lane represents an individual animal.

**Figure 4-7B**

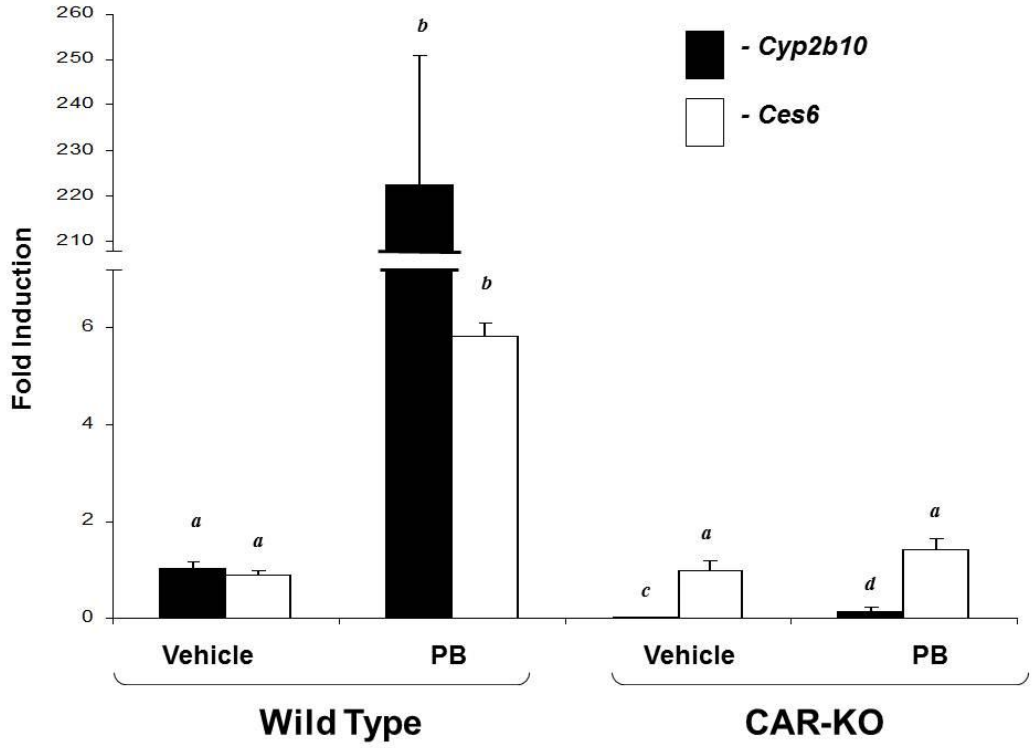


**Figure 4-7. Induction of *Ces6* and *Cyp2b10* gene expression by phenobarbital treatment in mouse liver is CAR-dependent.** Total RNA was isolated from the liver of wild type and CAR knockout mice ($n$=3) treated *i.p.* with saline (vehicle) or PB (100 mg/kg) for 4 days. (B) Real-time quantitative PCR analyses were performed to determine the expression of *Cyp2b10* and *Ces6*. All data are normalized to 18S levels and represent the average values ± SEM ($n=5$) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

**Figure 4-8A**

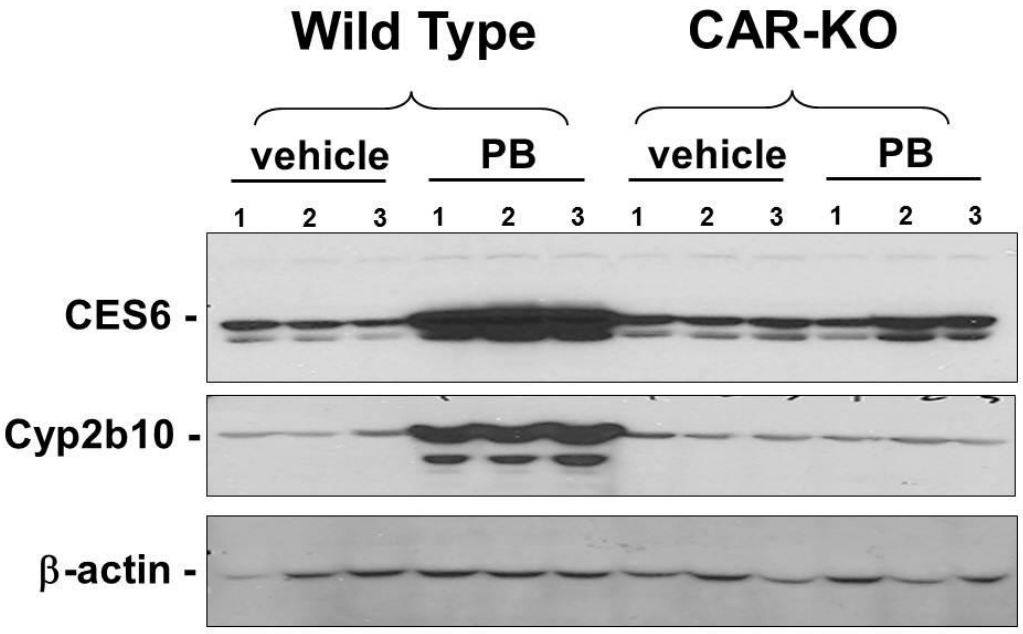


**Figure 4-8. Expression of Ces6 and Cyp2b10 protein is induced by phenobarbital in a CAR-dependent manner in mouse liver.** Microsomes were isolated from the livers of wild type and CAR-KO mice treated with saline (vehicle) or phenobarbital for 4 days (n = 3). (A) Western blot analysis was performed to determine the expression levels of Ces6, Cyp2b10 and β-actin protein, respectively. Each lane represents an individual animal.

**Figure 4-8B**

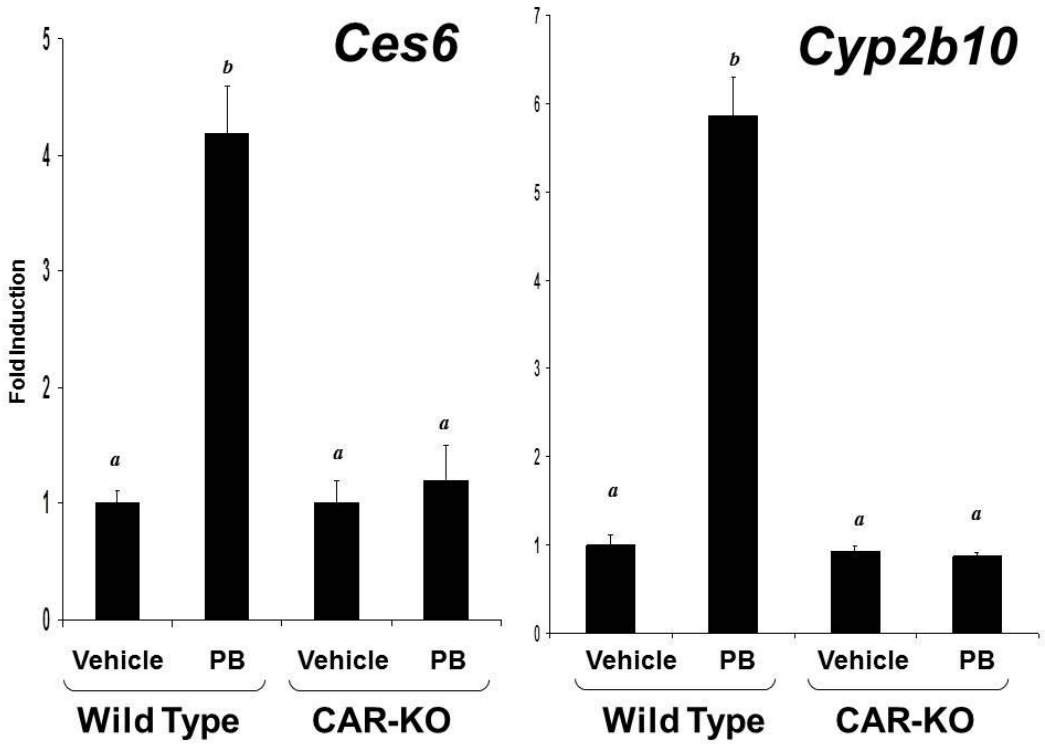


**Figure 4-8. Expression of Ces6 and Cyp2b10 protein is induced by phenobarbital in a CAR-dependent manner in mouse liver.** Microsomes were isolated from the livers of wild type and CAR-KO mice treated with saline (vehicle) or phenobarbital for 4 days (n = 3). (B) The results from (A) were quantified using scanning densitometry. All data are normalized to β-actin levels and represent the average values ± SEM (*n=3*) and expressed as fold induction over vehicle control. Letters different from each other indicate a statistical difference between treatment groups ($p<0.05$).

in duodenum (data not shown). These data indicate that the drug-inducible expression and activity of the Ces6 and Cyp2b10 gene products are regulated by both PXR and CAR nuclear receptor proteins. Taken together, these data lead to a model in which drug-inducible activation of intestinal carboxylesterase activity in intestine would be expected to increase the conversion of prodrugs to the active drug, thereby increasing the transport to portal vein and liver (Figure 4-9). In the liver, high levels of cytochrome P450 and carboxylesterase activity would be expected to further increase metabolism of co-administered drugs leading to increased prospects for drug-drug interaction in patients on combination therapy.

### *4.4 Discussion*

The first-pass effect is a phenomenon of drug metabolism whereby the concentration of a drug is greatly reduced before it reaches the systemic circulation. This effect is largely mediated by drug metabolizing enzymes and drug transporter proteins in the small intestine and liver of mammals. When administered in the active form, these tissues metabolize many drugs to such an extent that only a small amount of the active drug emerges from the liver to the rest of the circulatory system. Increasingly, prodrugs are being designed using a rational approach that takes advantage of the enzymes and transporter proteins in these tissues in such a manner that promotes their biotransformation and absorption following oral administration. Specifically, introduction of an ester group generally improves bioavailability due to increased transport. Ester-containing prodrugs including several

**Figure 4-9**

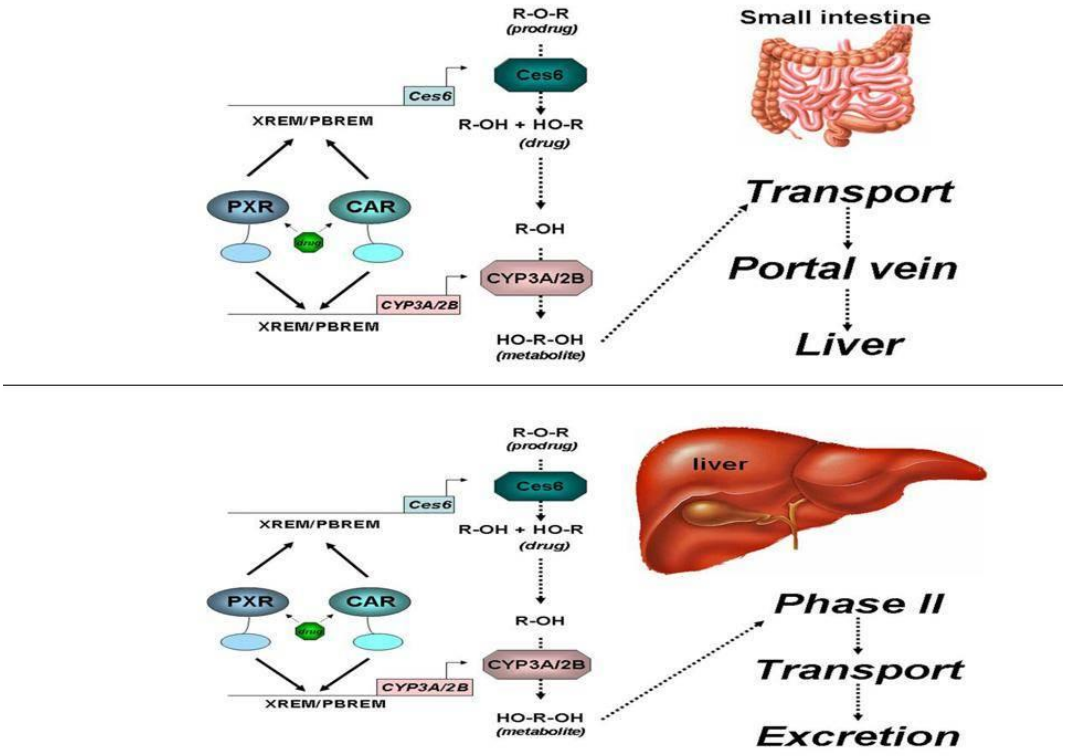


**Figure 4-9. Model of Coordinated PXR- and CAR-mediated Gene Activation in Liver and Intestine.** Activation of PXR and CAR in intestine produces elevated levels of Ces6 and cytochrome P450 drug metabolizing activity (*top panel*). This would be expected to accelerate conversion of prodrug to active drug, and increase uptake into the portal circulation. The liver would then mediate further uptake metabolism and excretion into bile and elimination in feces, or back into blood for eventual elimination through the kidney and in urine (*bottom panel*).

angiotensin-converting enzyme inhibitors, anti-tumor drugs, and narcotics are acted upon by carboxylesterase enzymes in this manner. In this regard, carboxylesterase enzymes are considered to be one of the major determinants of the metabolism and disposition of ester-containing drugs through their actions in liver and intestine.

Most of what is known regarding the liver- and intestine-enriched carboxylesterase enzymes (CES1 and CES2 sub-families) consists of characterization of their respective substrate specificities. Specifically, the CES1 sub-family of carboxylesterase enzymes mainly hydrolyzes substrates with small alcohol and large acyl groups. In contrast, the CES2 sub-family of carboxylesterase enzymes mainly hydrolyzes substrates with large alcohol and small acyl group. While much is known regarding their substrate selectivity, little is known regarding the regulation of expression of these important drug metabolizing enzymes in liver and intestine, though it is a topic of intense study in several laboratories.

Because expression of the prototypical PXR-target gene, Cyp3a11, was still detectable in duodenum when compared with that observed in jejunum or ileum (Figure 4-1), we chose to further analyze the expression of PCN-inducible genes in this particular tissue. We demonstrate here that treatment of mice with PCN, a known PXR activator, induces the expression of multiple genes in duodenum involved in the regulation of drug metabolism and disposition. It is important to note here that we did not observe regulation of CAR-target gene expression following treatment of mice with PB, a known indirect and phosphorylation-dependent activator of CAR, in the intestine, despite significant expression of the CAR gene itself in intestine. In contrast,

treatment of mice with the direct activating ligand of CAR, TCPOBOP, produced significant increases in the expression of CAR-target genes examined here. Therefore, it is now tempting to speculate that the two different modes of activation by these two CAR-activating compounds are responsible for the distinct CAR-mediated gene activation profiles in liver when compared with that observed in intestine. Alternatively, differences in the bioavailability of these two compounds *in vivo* following *i.p.* administration could in principle be responsible for the apparent disparate results observed in this study. Interestingly, deletion of *Pxr* produced significant increases in the level of *Ces6* gene expression in both duodenum and liver. While the trend was clearly toward elevated expression, the level of *Cyp3a11* gene expression did not quite reach statistical significance in duodenum in PXR-KO mice. Nonetheless, these data indicate a likely repressive role for non-liganded PXR protein in duodenum, similar to what was observed for Cyp3a11 in this study (Figure 4-4B) and to what has been previously reported by our group in liver tissue [28].

It is well established that relatively small increases in gene expression noted using microarray technology can sometimes translate into very big changes in protein levels. This is especially true with respect to genes that encode proteins that participate in drug metabolism pathways. Specifically, certain cytochrome P450 genes, as well as those encoding other drug metabolizing enzymes such as the glutathione S-transferase enzymes, are known to be highly regulated at the level of transcription. Indeed, among the PCN-inducible genes in duodenum reported here, several encode glutathione S-transferase enzymes (*Gstm1*, *Gstm3*, *Gstm6*, *Gsta2*, *Gsta4*, *Mgst1*, and

*Mgst2*), drug metabolizing enzymes Cyp2C (*Cyp2c55* and *Cyp2c29*) and Cyp3A (*Cyp3a25* and *Cyp3a11*) family members, many of which have been previously identified as PXR-target genes in liver [7, 17]. Interestingly, expression of the gene encoding epoxide hydrolase was induced in duodenum following treatment with PCN. Epoxide hydrolase is well known to be induced by compounds that produce electrophilic and oxidative stress via the Nrf2-Maf transcription factor complex [29], however, relatively little is known regarding the regulation of this gene by PXR in duodenum. Our observation of significant up-regulation of epoxide hydrolase by PXR agonist treatment in small intestine is in agreement with a recent report that utilized PXR activators in rat model systems [30]. More research will need to be conducted to verify this finding and determine its potential biological relevance to drug metabolism and disposition in mammals in both liver and intestine.

The Abca1 gene product functions as a cholesterol efflux pump in the cellular lipid removal pathway. Mutations in Abca1 have been associated with Tangier's disease and familial high-density lipoprotein deficiency in humans [31]. It is interesting that our analysis identified Abca1 as a PCN-inducible gene in duodenum since it has previously been identified as being down-regulated by PXR agonist treatment in transformed human liver cell lines as well as in rodent hepatocytes [32, 33]. Still other studies indicate that Abca1 is up-regulated in small intestine in mice in a PXR-dependent manner [34]. Another study indicates that treatment of intestinal cell lines Caco2 and Ls174T with the PXR agonist rifampicin induces Abca1 expression, but does not affect expression of this gene in liver cell lines [35]. Clearly, more

research is necessary to determine the molecular basis by which this important cholesterol efflux transporter is regulated differentially in hepatic versus intestinal tissue; however, our data are consistent with others that indicate that this gene is up-regulated in intestine following treatment with PXR agonists.

Our analysis also identified the acyl-CoA-binding protein (ACBP), or diazepam binding inhibitor (Dbi), as a PCN-responsive gene. The ACBP/Dbi gene encodes a 10-kDa intracellular protein that specifically binds acyl-CoA esters with high affinity. This small protein is expressed in most cell types at low levels; however, its expression is inducible by metabolic and xenobiotic signals through SREBP and PPARα signaling in hepatocytes, respectively [36]. Our data indicate that PXR also likely regulates the drug-inducible expression of this important gene in small intestine.

Other genes of note up-regulated in mouse intestine following treatment with PCN include 17-beta-Hydroxysteroid dehydrogenase type 11 (Hsd17b11), a member of the short-chain dehydrogenase/reductase family. The Hsd17b11 gene product is involved in the activation and inactivation of sex steroid hormones in liver and intestine. It is interesting to note that treatment of mice with the potent peroxisome proliferator Wy14 643 induced expression of this gene product in both liver and intestine [37], presumably through activation of PPARα. However, since several PPARα agonists are also PXR agonists, it is possible that induction of Hsd17b11 gene expression by treatment with Wy14 643 occurs, in part, through activation of PXR by this compound.

Another PCN-inducible gene detected in duodenum by our analysis is pyruvate dehydrogenase kinase, isozyme 4 (Pdk4). This gene encodes a member of the PDK protein kinase family that inhibits the pyruvate dehydrogenase complex by phosphorylating one of its subunits. Activation of this gene by GR, PPAR-delta, and FXR agonists contributes to the regulation of glucose metabolism in several tissues [38, 39].

The nuclear receptors PXR and CAR were originally identified and characterized as 'xenobiotic sensors', however, more recent research indicates a wider role for these two receptors in regulation of the response to metabolic and nutritional stress (reviewed in [40]). In any case, taken together our data indicate that activation of PXR-target gene expression in intestine regulates the expression of genes involved in modulating drug metabolism, the response to oxidative stress, as well as the disposition of steroids, glucose, and cholesterol homeostasis. Future research should seek to unravel the molecular basis for the differential interaction between nuclear receptor signaling and gene activation pathways in a tissue-selective manner. Additional whole animal studies should be performed to test our model that would include carboxylesterase activity assays, as well as monitoring prodrug and drug plasma levels following administration of PXR and CAR activators *in vivo*. Additional studies should be performed using primary hepatocytes and immortalized cell lines to determine whether the signaling pathways investigated here are evolutionarily conserved in humans. Together, these issues represent interesting research opportunities for the future.

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# Chapter 5: Pregnane X Receptor is Targeted by the Ubiquitin-proteasome Pathway in a Signal-dependent Manner

### *5.1 Introduction*

Adverse drug reactions (ADRs) are among the top ten leading causes of death in the world. ADRs are also a major cause of death among hospitalized patients in the United States. It is well known that the majority of ADRs result directly from the unexpected abnormal metabolic handling of drugs by patients whose hepatic drug metabolizing enzymes (DMEs) have been induced or inhibited. A substantial proportion of ADRs are due to the occurrence of disease-drug interactions in which systemic infection and acute inflammation can seriously impair drug metabolism in patients, leading to serious and potentially lethal consequences. Currently, the molecular basis for this impairment is not known.

The pregnane x receptor (PXR, NR1I2) is a member of the nuclear receptor (NR) superfamily of ligand-activated transcription factors. In mammals, PXR is expressed at high levels in the liver and the intestine, major organs that are important in xenobiotic biotransformation [1]. The expression of PXR has also been detected in both normal and neoplastic breast tissue, as well as in peripheral blood mononuclear cells [2, 3]. As a heterodimer with the nuclear receptor retinoid x receptor alpha (RXR$\alpha$, NR2B1), PXR regulates the expression of its target genes through binding to specific PXR response elements. Numerous studies have characterized PXR response elements, which have shown that PXR binds to two copies of the consensus nuclear receptor binding motif AG(G/T)TCA arrayed as a direct repeats separated by 3 or 4

nucleotides (DR-3 and DR-4) or everted repeats separated by 6 or 8 nucleotides (ER-6 and ER-8) [1]. A recent study using chromatin immunoprecipitation (ChIP)-on-chip and ChIP-sequencing (ChIP-Seq) identifies the most frequent PXR DNA-binding motif is the AGTTCA-like DR-4. Surprisingly, there are also high motif occurrences with spacers of a periodicity of 5 nucleotides, forming a novel DR-(5n+4) pattern for PXR binding [4].

PXR is now well established to function as a positive regulator of the expression of genes that encode key DMEs and drug transporter proteins involved in the uptake, metabolism and elimination of xenobiotic compounds in the liver and the intestines [5-8]. However, recent research describes the negative regulation of several key biochemical activities by ligand-mediated activation of PXR in the liver and the intestine including gluconeogenesis, lipid metabolism, ketogenesis, and the inflammatory response [9]. For example, activation of PXR by rifampicin, the prototypical activator of human PXR, suppresses the expression of several key inflammatory response genes including IL-1β, TNFα… [10].

The ubiquitin-proteasome degradation pathway is the major system for selective degradation of proteins in eukaryotic cells. Targeted proteins are covalently modified with one or several molecules of the highly conserved 76 amino acid ubiquitin protein. Targeted proteins are subsequently degraded by the 26S proteasome complex, a large multisubunit protease [11]. Conjugation of ubiquitin to target proteins proceeds via a three-step cascade mechanism: (1) initially, a high-energy thioester bond is formed between the C terminus of ubiquitin and the active site cysteine on ubiquitin-activating

E1 enzyme in an ATP-dependent manner; (2) the activated ubiquitin is then trans-esterified to a conserved cysteine on any one of the ubiquitin-conjugating E2 enzymes; (3) the ubiquitin E3 ligase interacts with both E2 and the substrate, and the ubiquitin bound E3 ligase targets the ubiquitin to the protein substrates, which are then subjected to proteasome degradation into peptides. Ubiquitin can be removed from protein substrates through the action of deubiquitinating enzymes, which can serve to reverse the effects of ubiquitination (Figure 5-1).

The ubiquitin conjugation process can result in mono-ubiquitination or multi-mono-ubiquitination through attachment of a single ubiquitin molecule(s) to lysine residues within the target protein. Poly-ubiquitin chains can also be formed through further attachment of ubiquitin molecule to any of the seven lysines within ubiquitin itself (Lys-6, Lys-11, Lys-27, Lys-29, Lys-33, Lys-48, and Lys-63) or to the N-terminal methionine [12]. The fate of an ubiquitinated protein is thought to be determined by the type of ubiquitin linkage associated with it. Formation of Lys-48 and Lys-11-linked ubiquitin chains targets the substrate protein for degradation by the 26S proteasome. Lys-63-linked chains usually mediate the recruitment of binding partners, which can lead to a variety of non-proteolytic biological activities of the substrate, namely activation of nuclear factor-κB (NF-κB), orchestration of different steps during DNA repair, or targeting the modified protein to the lysosome. Other ubiquitin chains, such as Lys-6 or Lys 29-linked chains, have been detected *in vitro* or *in vivo*, but their significance in cellular regulation has not yet been established [13].

**Figure 5-1**

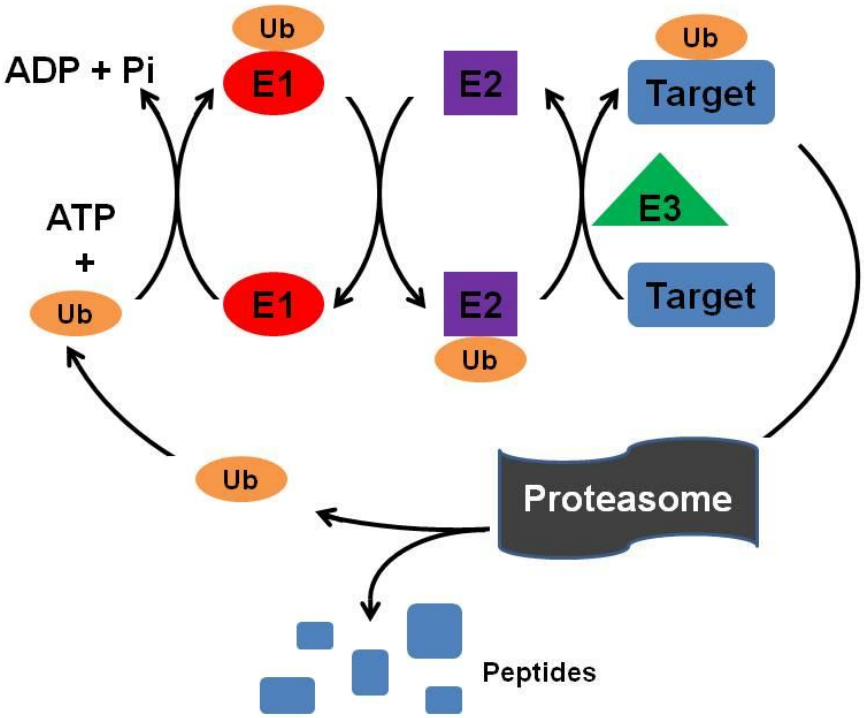


**Figure 5-1. The Ubiquitin-proteasome Pathway.** The process initiates through an APT-dependent activation of free ubiquitin by an ubiquitin-activating enzyme E1. The activated ubiquitin is then transferred to an ubiquitin-conjugating enzyme E2 and finally to an ubiquitin ligase E3. The ubiquitin bound E3 ligase targets the ubiquitin to the protein substrate, which is subjected to proteasome degradation into peptides. Ubiquitin is deconjutated by deconjutating enzymes and recycled for next round of pathway.

In order to maintain the cellular activity through a balance between the synthesis and breakdown of signaling molecules, degradation of regulatory proteins by the ubiquitin-proteasome pathway is an important mechanism for the tight control of diverse cellular processes, ranging from gene transcription to cell cycle progression [14, 15]. Aberrations within protein substrates undergoing ubiquitin-proteasome degradation are implicated in several diseases including Alzheimer's disease and cancer [16, 17]. Transcription factors are among the proteins regulated by the ubiquitin-proteasome pathway, and included in this group are members of the NR superfamily [18]. Ligand-dependent regulation by the ubiquitin-proteasome system has been demonstrated for several members of the NR family, including estrogen receptor alpha (ERα, NR3A1), retinoic acid receptor alpha (RARα, NR1B1) and gamma (RARγ, NR1B3), peroxisome proliferator-activated receptor gamma (PPARγ, NR1C3) [19-21].

Our laboratory has recently shown that PXR exists in cells as a phosphoprotein and that activation of PKA signaling interfaces with PXR to inhibit its transcriptional activity in hepatocytes [22, 23]. Here, we identify PXR as the molecular target of ubiquitin. Moreover, we show that ubiquitination of PXR is stimulated in cells by treatment with cyclic-AMP and activation of the MEKK1 signaling pathway, suggesting distinct regulation of PXR activity by metabolic- and inflammatory-mediated signaling. Interestingly, inhibition of the proteasomal degradation pathway and increased ubiquitination of PXR represses rifampicin-inducible PXR transactivation capacity in an engineered PXR reporter gene

assays. Taken together, this novel data provides a plausible and testable hypothesis for how inflammatory- and cyclic AMP/PKA-mediated signaling pathways selectively repress the drug-inducible expression and activity of hepatic drug metabolizing and drug transporter activities in the liver and the intestine.

### *5.2 Materials and Methods*

***Compounds and Plasmids.*** Unless otherwise stated, all chemical compounds were purchased from Sigma (St. Louis, MO). Plasmids pRK5-HA-Ubiquitin-WT, KO, K48R and K63R were purchased from Addgene (Cambridge, MA). His-tagged-ubiquitin constructs were subcloned into the pcDNA4/Hismax A expression vector (Invitrogen) at EcoRI and NotI restriction sites. The pcDNA4/Hismax-Ubiquitin-K48, 63R expression vector was generated from pcDNA4/Hismax-Ubiquitin-K48R by site-directed mutagenesis with use of the QuikChange Mutagenesis system (Stratagene, La Jolla, CA). Primer sequences used for site-directed mutagenesis are as follows: forward primer (5'-GCTGTCTGATTACAACATTCAGAGGGAGTCCACCCT-3'), reverse primer (5'-AGGGTGGACTCCCTCTGAATGTTGTAATCAGACAGC-3').

***Cell-based Ubiquitination Assays.*** HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM L-glutamine, 100 U/ml penicillin/streptomycin, and 10% fetal calf serum. For transfection assays, HeLa cells were grown for 24 hours until 80% confluence. Cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen, Carlsbad, CA). 24 hours post-transfection, cells were treated as indicated for an additional 24 hours. 48 hours

after transfection, cells were washed with phosphate-buffered saline and harvested with 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 3000g for 15 minutes. The cleared cell lysates were mixed with $Ni^{2+}$-linked agarose (QIAGEN, Valencia, CA) that had been prewashed with cell lysis buffer. The mixture was incubated for 2 hours on a rotator at room temperature and centrifuged for 2 minutes at 1000 rpm to gather the beads. The beads were washed once in lysis buffer, three times in wash buffer (8 M urea, 100 mM sodium chloride, 50 mM sodium phosphate buffer, pH 6.3), and once in phosphate-buffered saline. The beads were resuspended in SDS-PAGE gel loading buffer and boiled for 5 minutes. Samples were resolved on 10% SDS-PAGE. The gel was transferred to polyvinylidene difluoride membrane (Millipore Bioscience Research Reagents) using standard methods, and probed with H-11 monoclonal anti-PXR antibody (Santa Cruz Biotechnology, Inc. Antibodies) to detect the ubiquitinated form of PXR. Immunodetection was performed using the Pierce ECL western blotting substrate (Thermo Scientific) according to the protocol provided by the manufacturer.

***Hepatocyte Culture and Immunoprecipitation of Human PXR Protein.***

Primary cultures of human hepatocytes were purchased (Invitrogen). 48 hours after plating, hepatocytes were treated with vehicle (0.1% DMSO), 10 ng/ml TNFα, or TNFα plus rifampicin (10 μM), proteasome inhibitor MG132 (25 μM), and TNFα plus MG132. 24 hours after drug treatment, cells were lysed by sonication in a buffer composed of 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 1 mM EDTA, 1% Triton X-100,

and 1 × protease inhibitors (Thermo Fisher Scientific, Waltham, MA). Cell lysates were precleared with 20 μl of immobilized protein A (Repligen, Waltham, MA). Immunoprecipitation of the human PXR protein was accomplished by using a custom polyclonal antibody directed against the human PXR ligand-binding domain. Free immune complexes were captured with immobilized protein A and washed three times with lysis buffer. After SDS-PAGE, the protein was transferred to polyvinylidene difluoride membrane (Millipore Bioscience Research Reagents, Temecula, CA) that was probed with a monoclonal anti-PXR antibody (Santa Cruz Biotechnology, Inc. Antibodies). Immunodetection was performed by using the Pierce ECL Western blotting substrate (Thermo Fisher Scientific) according to the protocol provided by the manufacturer.

***Transient Transfection and Reporter Gene Analysis.*** The reporter gene assays were performed as described previously [24]. In brief, CV-1 cells were plated in 96-well plates at a density of 7000 cells per well. After 24 hours the cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. The PXR transactivation assays were performed by transfecting cells with CMV-β-galactosidase (20 ng), XREM-LUC or NF-κB-LUC (20 ng), pSG5-hPXR (5 ng), His-ubiquitin (10 ng), and pBluescript was added to achieve 110 ng of total DNA per well. 24 hours post-transfection cells were treated with vehicle (0.1% DMSO) or drug (10 mM rif, 100 mM ALLN, 25 mM MG132, 10 mM LactaC, and 10 ng/ml TNFα) for additional 24 hours. Luciferase activities were determined by using a standard luciferase assay system (Promega, Madison, WI).

β-Galactosidase activities were determined using an o-nitrophenyl-β-d-galactopyranoside (ONPG) assay. For the ONPG assay, 110 mg of ONPG was dissolved in 100 ml of 0.1 M $NaHPO_4$ buffer, which was made by mixing 6.84 ml of 1 M $Na_2HPO_4$, 3.16 ml of 1 M $NaH_2PO_4$, and 90 ml of $H_2O$. 20 μl of cell lysate and 200 μl of ONPG buffer were mixed, and the mixture was incubated at 37 ℃ for 30-60 minutes and read at 420 nm.

### *5.3 Results*

***PXR is the Molecular Target of Ubiquitin-proteasome Degradation.*** Because past research from our laboratory identified a strong interface between PKA signaling and PXR activity [22, 25, 26], and because several members of the NR family have been previously identified to be regulated by the ubiquitin-proteasome pathway [19-21], we sought to determine whether PXR is also a regulated molecular target of the ubiquitin system. To initiate these studies, we first tested the effects of proteasome inhibitors on levels of exogenously expressed FLAG-tagged human PXR expression in the human hepatoma immortalized cell line-HepG2 cells. The levels of PXR protein were increased by treatment with all three proteasome inhibitors examined including ALLN (Acetyl-L-Leucyl-L-Leucyl-L-Norleucinal), MG132 (Benzyloxycarbonyl-L-Leucyl-L-Leucyl-L-Leucinal), and lactacystin (N-Acetyl-L-Cysteine, S-[2R,3S,4R]-3-Hydroxy-2-[(1S)-1-Hydroxy-2-Methylpropyl]-4-Methyl-5-Oxo-2-Pyrrolidinecarbonyl]). Not much change was observed when cells were treated with rifampicin alone, or in combination with proteasome inhibitors (Figure 5-2A).

Similar results were also observed in HeLa cells (Figure 5-2B). These results indicate that PXR is the likely target of the 26S proteasomal degradation machinery.

Since the 26S proteasome recognizes and degrades proteins that are conjugated with poly-ubiquitin chains, we next sought to determine whether we could detect poly-ubiquitination of PXR in transfected cultured cell lines. A combination of expression vectors encoding the ubiquitin and PXR proteins were employed using a cell-based transient transfection based strategy. Co-expression of 6×-histidine-tagged PXR and ubiquitin in HeLa cells allowed us to exam whether PXR is the target of ubiquitin. Cultured HeLa cells expressing both PXR and ubiquitin expressed a protein recognized by the anti-PXR antibody that corresponds to the expected size of mono-ubiquitinated PXR protein. Treatment with rifampicin decreased the expression of mono-ubiquitinated PXR to a small extent (Figure 5-3, lanes 5 and 6). As expected, treatment of HeLa cells over expressing both PXR and ubiquitin with the proteasome inhibitor MG132 increased ubiquitination of PXR (Figure 5-3, lanes 7 and 8). This data further supports the conclusion that PXR is the molecular target of ubiquitin-proteasome pathway.

***Poly-ubiquitination of the PXR Protein is Regulated by Cyclic AMP and MEKK Signaling Pathways.*** We next treated cells with activators of pivotal signal transduction pathways to determine if poly-ubiquitination of PXR is regulated by cell signaling. Notably, treatment with 8-bromo-cyclic AMP, the cell-permeable activator of the PKA signaling pathway increased the level of PXR ubiquitination, while

**Figure 5-2A**

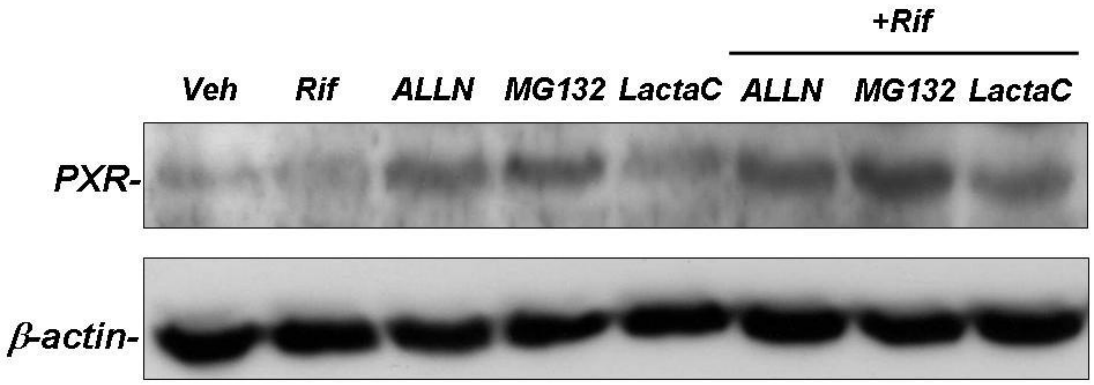

**Figure 5-2B**

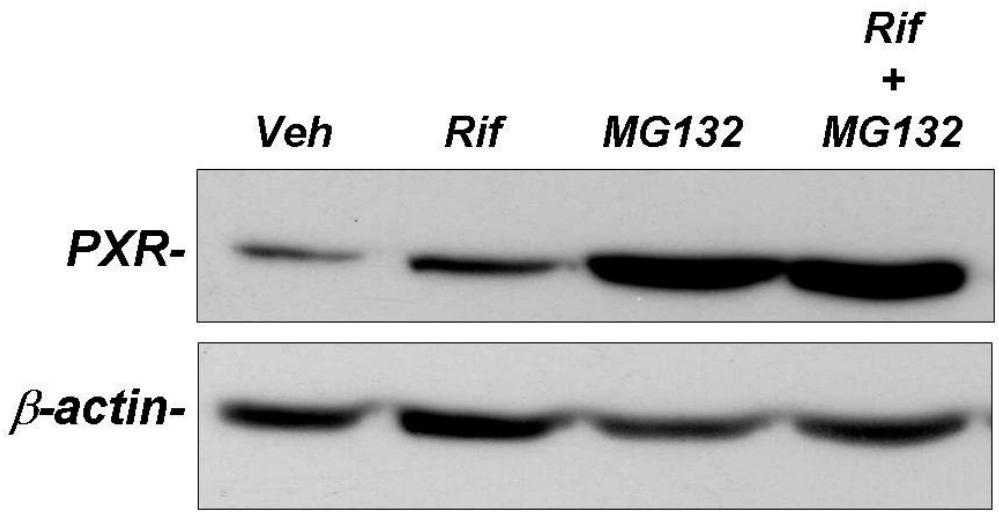


**Figure 5-2. Expression of PXR Protein is Increased by Proteasome Inhibitors in Cells.** The plasmid encoding FLAG-tagged human PXR protein was transfected into HepG2 (Figure 5-2A) or HeLa (Figure 5-2B) cells. 24 hours post-transfection, cells were treated as indicated for an additional 24 hours. Total cell extract was subjected to SDS-PAGE and western blot analysis was performed to determine the expression levels of human PXR and β-actin protein, respectively.

**Figure 5-3**

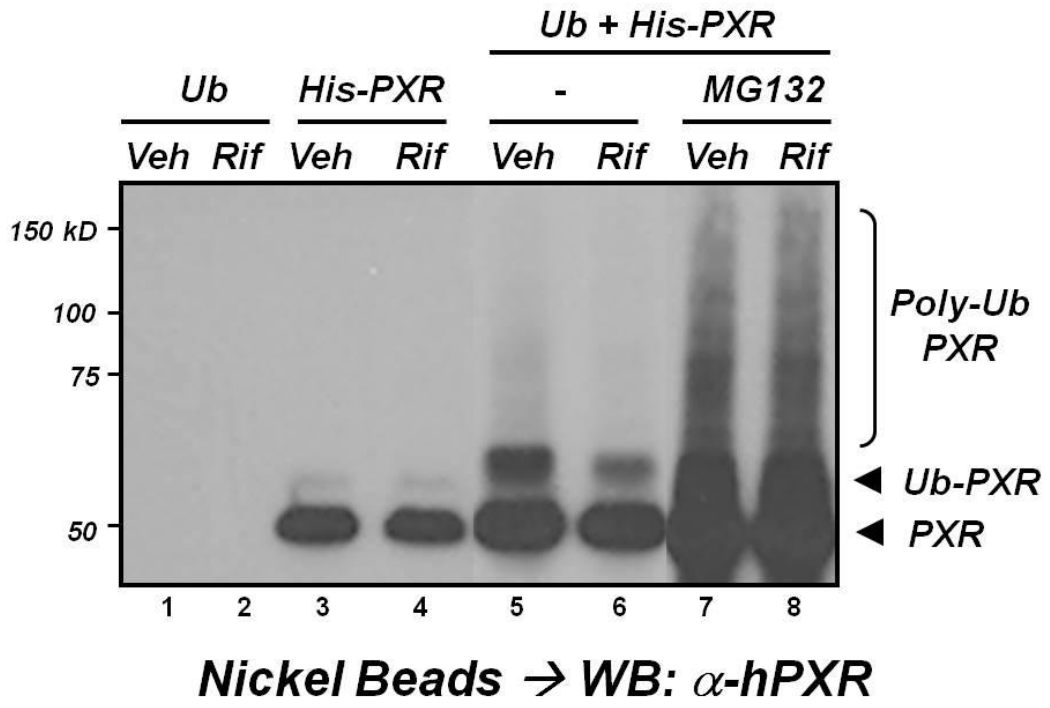


**Figure 5-3. PXR is Ubiquitinated in Cells.** The plasmid encoding the 6x-His-tagged PXR protein was transfected alone or together with an expression vector encoding ubiquitin into HeLa cells. 24 hours post-transfection, cells were treated as indicated for an additional 24 hours. Total cell extract was subjected to purification using nickel-linked agarose beads, followed by SDS-PAGE and western blotting using a monoclonal antibody against the human PXR protein.

treatment with 8-bromo-cyclic GMP had no effect on it (Figure 5-4, lanes 5 and 6). This is a significant discovery in light of the fact that the proteasome activity is increased by PKA-mediated phosphorylation [27]. Our laboratory has recently shown that PXR is also phosphorylated by PKA, and that PKA signaling interfaces with PXR to inhibit its transcriptional activity in hepatocytes [22, 23]. Interestingly, forced expression of constitutively active kinases MEKK1 (activates JNK) increased PXR poly-ubiquitination, while forced expression of MEK1 (activates ERK) and MEK3 (activates p38) had no effect on ubiquitination of PXR (Figure 5-4, lanes 7-9). TNF$\alpha$ treatment is known to induce MEKK1 activation [25]. The question of whether ubiquitination of PXR affects transcription of DMEs therefore becomes important to address.

***Cyclic-AMP Mediates Poly-ubiquitination of PXR via neither Lys-48 nor Lys-63-linked Ubiquitin Chains.*** To directly assess the role of Lys-48 and Lys-63-linked ubiquitin chains in cyclic-AMP mediated poly-ubiquitination of PXR, different ubiquitin mutants containing lysine-to-arginine mutations were employed to prevent the ubiquitin chain formation on indicated lysine residues. Treatment of HeLa cells co-expressing both PXR and KO form of ubiquitin (ubiquitin with all lysines mutated to arginine residues) with 8-bromo-cyclic AMP decreased poly-ubiquitination of PXR compared to the one with WT form of ubiquitin (Figure 5-5, lanes 5 and 7). Importantly, cells co-expressing PXR and three other mutant forms of ubiquitin, including K48R (ubiquitin with lysine 48 mutated to arginine), K63R (ubiquitin with

**Figure 5-4**

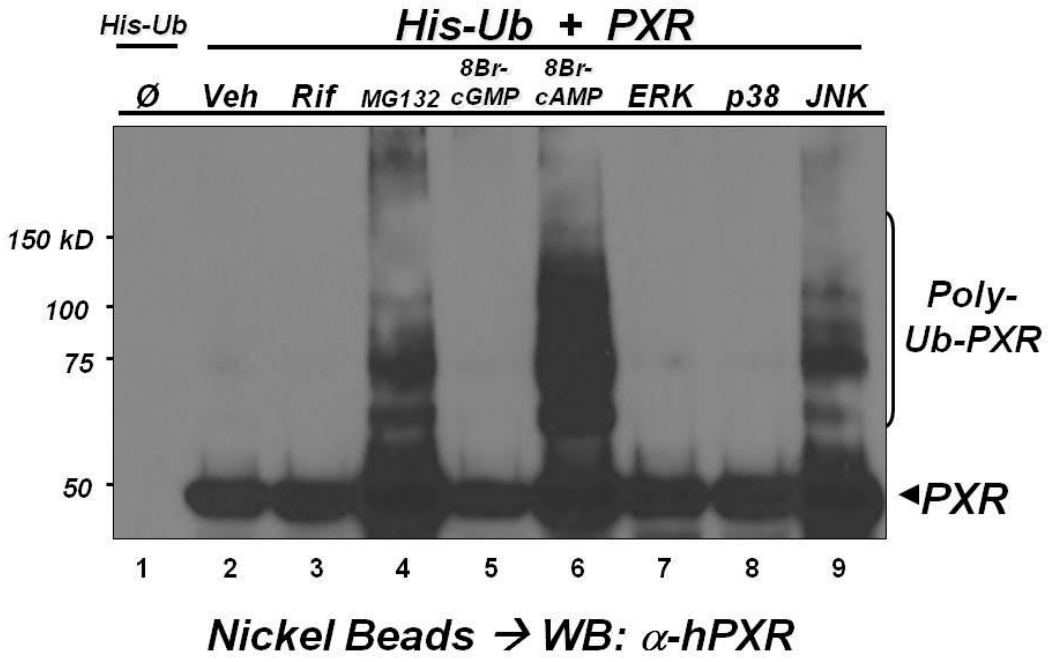


**Figure 5-4. Poly-ubiquitination of PXR Protein is Stimulated by Cyclic-AMP and Constitutively Active MEKK Signaling.** The plasmid encoding the 6x-His-tagged ubiquitin protein was co-transfected into the HeLa cells with the expression vector encoding the PXR protein and activators of the ERK, p38, and JNK signaling cascades. 24 hours post-transfection cells were treated as indicated for an additional 24 hours. Total cell extract was subjected to purification using nickel-linked agarose beads, followed by SDS-PAGE and western blotting using a monoclonal antibody against the human PXR protein.

**Figure 5-5**

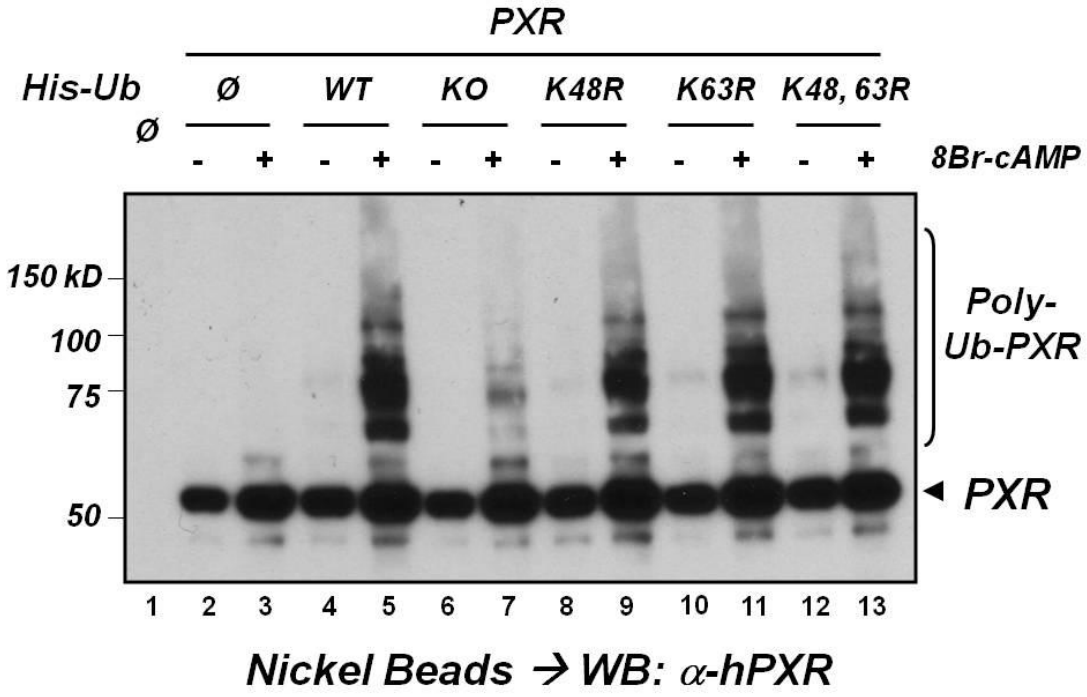


**Figure 5-5. Cyclic-AMP Mediates Poly-ubiquitination of PXR via neither Lys-48 nor Lys-63-linked Ubiquitin Chains.** The plasmid encoding PXR protein was co-transfected into the HeLa cells with an expression vector encoding 6x-His-tagged wild type ubiquitin (WT), ubiquitin with all lysines mutated to arginine residues (KO), ubiquitin with lysine 48 mutated to arginine (K48R), ubiquitin with lysine 63 mutated to arginine (K63R), or ubiquitin with lysines 48 and 63 mutated to arginines (K48, 63R). 24 hours post-transfection cells were treated as indicated for an additional 24 hours. Total cell extract was subjected to purification using nickel-linked agarose beads, followed by SDS-PAGE and western blotting using a monoclonal antibody against the human PXR protein.

lysine 63 mutated to arginine), and K48, 63R (ubiquitin with lysines 48 and 63 mutated to arginines) still exhibited poly-ubiquitin chain formation on PXR protein (Figure 5-5, lanes 9, 11, and 13). This data indicates that other lysine residues rather than K48 or K63 within ubiquitin protein may be involved in cyclic-AMP mediated poly-ubiquitination of PXR.

***Ubiquitination of the PXR Protein is Increased by Rifampicin together with TNFα.*** We next used primary cultures of human hepatocytes in order to determine whether treatment with proteasome inhibitor MG132, TNFα, or co-treatment with TNFα and rifampicin modulates ubiquitination of the PXR protein. Co-treatment of human hepatocytes with rifampicin and TNFα dramatically increases PXR ubiquitination as detected using immunoprecipitation followed by western blotting (Figure 5-6, lane 3). As expected, treatment with MG132 increased ubiquitination of PXR (Figure 5-6, lane 4), while co-treatment with TNFα and MG132 further increased PXR ubiquitination (Figure 5-6, lane 5). This data demonstrates that the PXR protein is likely modified by ubiquitin in hepatocytes following co-treatment with TNFα and rifampicin, an inflammatory cytokine and PXR activator, respectively.

***Inhibition of the Proteasomal Degradation Pathway Abolishes PXR Transactivation Capacity.*** In order to determine the effect of inhibition of the proteasomal degradation pathway on PXR activity, we used a reporter gene approach with the xenobiotic response enhancer element (XREM) from the CYP3A4 promoter. This enhancer element fused to the luciferase reporter gene is well known to bind PXR,

**Figure 5-6**

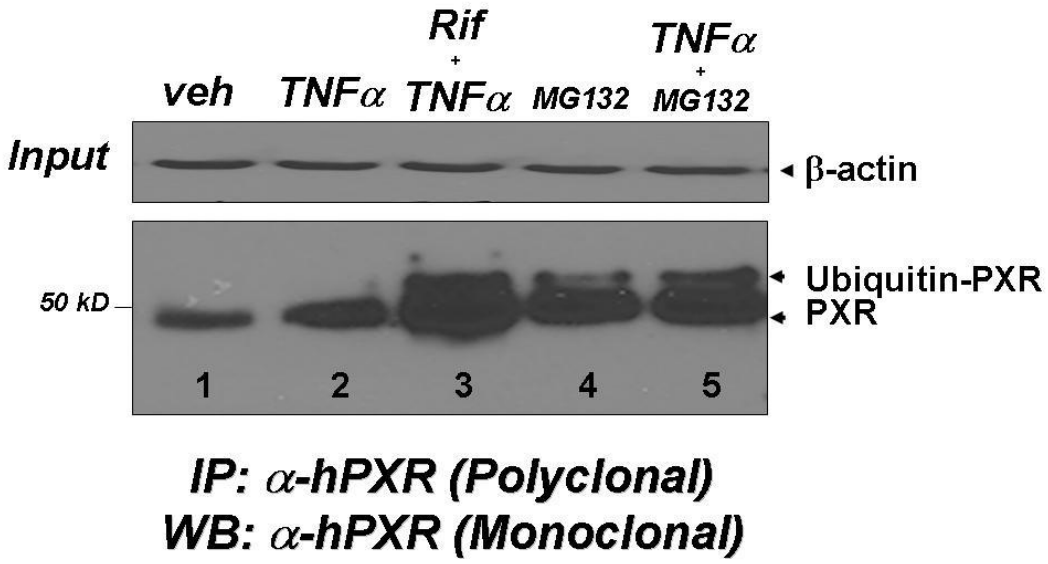


**Figure 5-6. Co-treatment of Hepatocytes with Rifampicin and TNFa Increases Ubiquitination of Human PXR Protein.** Primary cultures of human hepatocytes were treated with vehicle (0.1% DMSO), TNFα (10 ng/ml), or TNFα plus rifampicin (10 uM), proteasome inhibitor MG132 (25 uM), and TNFα plus MG132 for 24 hours. Cell extracts were subjected to immunoprecipitation with a polyclonal antibody that recognizes human PXR followed by SDS-PAGE and western blotting using a monoclonal antibody against the human PXR protein (bottom panel). Equal loading was insured using and aliquot of whole cell lysate and immunoblotting with β-actin (top panel).

and we therefore used this reporter gene as a marker for PXR activity in transfected CV-1 cells, a standard cell line for determining PXR reporter gene activity. Pharmacological inhibition of the proteasomal degradation pathway with three proteasome inhibitors ALLN, MG132 and lactacystin abolished PXR transactivation of the CYP3A4 promoter in reporter gene transfected CV-1 cells (Figure 5-7). 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 proxisome proliferator-activated receptor gamma (PPARγ, NR1C3) and liver x receptor alpha/beta (LXRα/β, NR1H3/NR1H2) [28, 29].

***Ubiquitination of PXR Represses Rifampicin-inducible PXR Transactivation Capacity.*** Our recent studies have shown that SUMOylated form of PXR protein represses NF-kB activity, but has little effect upon PXR-mediated CYP3A4 gene activation [30]. We next sought to determine the biological effect of forced expression of ubiquitin and treatment with TNFα on PXR activity using a reporter gene approach. When the PXR transfected cells were treated with rifampicin alone, the XREM reporter gene activity was induced approximately seven-fold. Co-treatment of cells with TNFα repressed activity of the reporter gene to a very modest extent. However, when ubiquitin was co-expressed together with PXR, the reporter gene activity was significantly lower when cells were co-treated with rifampicin and TNFα (Figure 5-8). Similar experiments using the NF-κB reporter gene revealed that forced expression of ubiquitin did not appreciably affect the ability of PXR to repress

**Figure 5-7**

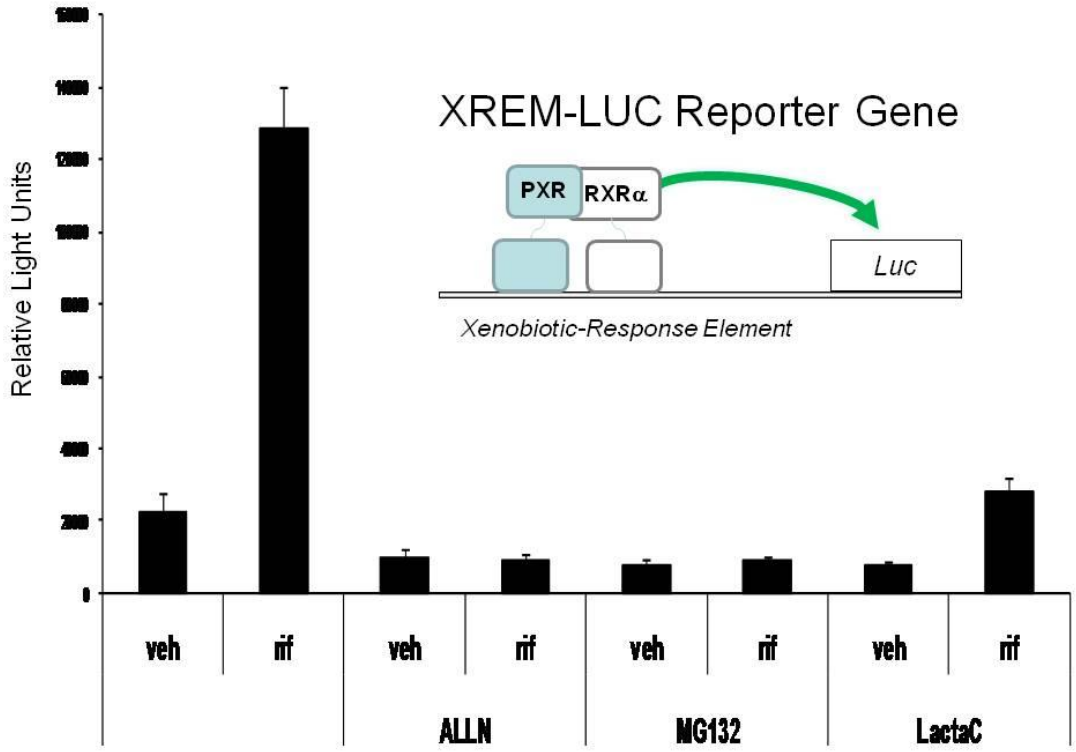


**Figure 5-7. Inhibition of the Proteasomal Degradation Pathway Abolishes PXR Transactivation Capacity in Transfected CV-1 Cells.** CV-1 cells were transfected with an XREM-luciferase reporter gene with plasmid encoding PXR protein. 24 hours post-transfection cells were treated as indicated for an additional 24 hours. Luciferase activity data was determined and reported as fold-induction ± S.E.M.

**Figure5-8**

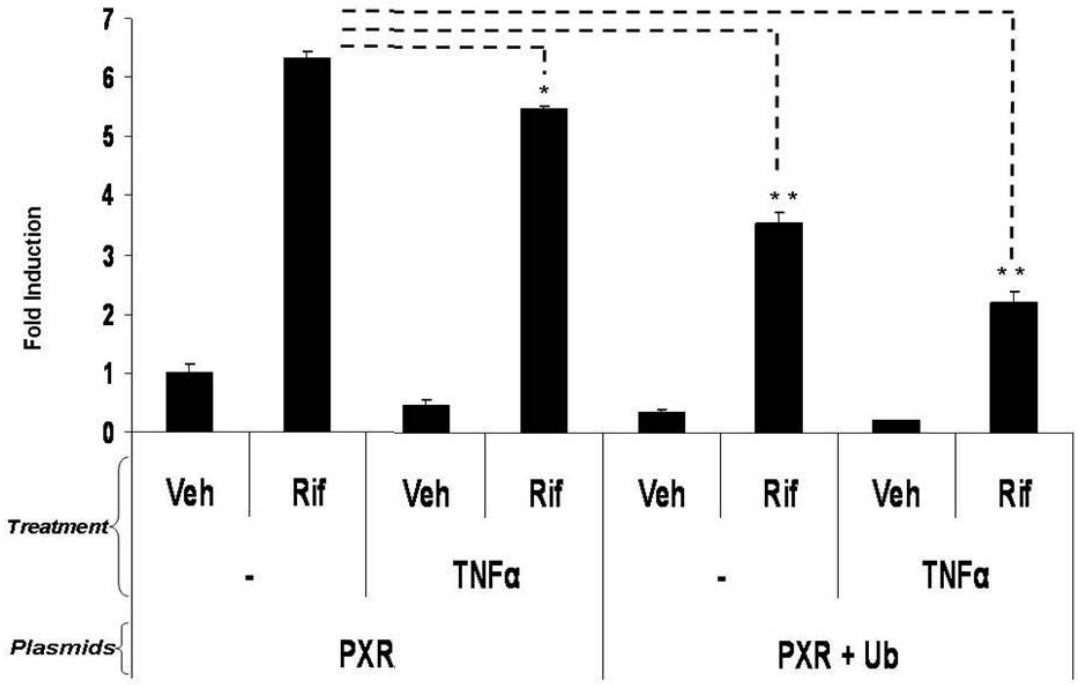


**Figure 5-8. The Biological Effect of Ubiquitin Modification of Human PXR on PXR Activity.** CV-1 cells were transfected with an XREM-luciferase reporter gene together with PXR alone, or PXR and ubiquitin. 24 hours post-transfection cells were treated as indicated for an additional 24 hours. Luciferase activity data was determined, normalized to β-glactosidase and reported as fold-induction ± S.E.M. （* = $p < 0.01$; ** = $p < 0.001$）.

NF-κB activity in the presence of TNFα (data not shown). This data reveals that ubiquitination of PXR protein may selectively clears the promoters of DMEs and other PXR target genes, therefore repressing their activity.

### *5.4 Discussion*

The proteome is in a dynamic state of synthesis and degradation, which controls the concentration of many proteins. Over 25 years ago, eukaryotic cells were shown to contain a highly specific system for the selective degradation of short-lived proteins. This system is known as the ubiquitin-proteasome pathway. In this pathway, proteins are targeted for degradation through covalent modification by a highly conserved protein named ubiquitin, while the proteasome is a giant cellular organelle with protease activities that degrades intracellular proteins in an ATP-dependent manner. Not only does the ubiquitin-proteasome degradation remove abnormal proteins that may be misfolded, aged, or damaged, it also plays an important role in numerous cell processes, including cell cycle progression, signal transduction and transcriptional regulation. NR proteins comprise a large superfamily of ligand-activated transcription factors with forty-eight members in the human genome [31]. NR-mediated transcriptional regulation is subject to multiple levels of control, including changes in chromatin structure within gene promoters and regulation of receptor and cofactor levels by the ubiquitin-proteasome pathway [32, 33]. Previous studies have shown that mouse PXR interacts with suppressor for gal1 (SUG1), which belongs to the 19S regulatory subunit of the 26S, in a progesterone-dependent manner [34]. In our recent experiments, human PXR ligand binding domain is also found to

interact with SUG1 in a yeast two hybrid screening. Here, we identify that PXR is the molecular target of ubiquitin. Inhibition of the proteasomal degradation pathway and ubiquitination of PXR represses rifampicin-inducible PXR transactivation capacity in reporter gene assays. This data provides an additional link between NR-mediated gene transcription and the ubiquitin-proteasome pathway.

Rapid degradation of a number of NR members by the ubiquitin-proteasome is correlated to their phosphorylation state. Phosphorylation is thought to signal substrate recognition by the enzymes in the ubiquitination pathway. Progesterone receptor is phosphorylated by mitogen activated protein kinase (MAPK) and degraded via the ubiquitin-proteasome pathway in a ligand-dependent manner [35]. Phosphorylation of retinoic acid receptor gamma 2 (RARγ2) by p38 MAPK leads to its degradation in response to retinoic acid [36]. Furthermore, hyperphosphorylation of peroxisome proliferator-activated receptor alpha (PPARα) induces its transcriptional activity which is accompanied by stabilizing the protein [37]. Our lab has recently identified that PXR exists as a phosphoprotein *in vivo* and that its phosphorylation status is modulated by the activation of PKA signaling [22, 23]. In the current studies, we demonstrate that ubiquitination of PXR protein can be stimulated in cells by treatment with cyclic-AMP, suggesting cross-talk between phosphorylation and ubiquitnation, two post-translational modifications of PXR through metabolic-mediated signaling.

The mitogen activated protein kinases (MAPKs) are a family of signal transduction proteins that convert extracellular signals, such as stresses and growth

factors, to the activation of intracellular pathways. Their activity is regulated through a module of sequentially acting cytoplasmic kinases composed of a MAP kinase, a MEK (MAP kinase kinase), and a MEKK (MEK kinase) [38]. Three subfamilies of MAP kinases are well characterized, including ERKs (extracellular signal-regulated protein kinases), the p38 MAP kinases, and JNKs (c-jun N-terminal kinases) [39]. Previous studies have shown that the PHD domain of MEKK1, a RING finger-like structure, exhibits E3 ubiquitin ligase activity and mediates ubiquitination and degradation of ERK1/2 [40]. In the current report, we demonstrate that forced expression of constitutively active kinase MEKK1, which activates JNK signaling, increases PXR poly-ubiquitination. MEKK1 may also act as an E3 ligase in this case to regulate PXR ubiquitination. TNFα treatment has been shown to induce MEKK1 activation [25]. On the other hand, inflammatory cytokines are also well known to repress drug-inducible expression of hepatic DMEs [41]. Our data supports the idea that inflammatory cytokine signaling in the liver increases ubiquitination of PXR protein. Co-treatment of cells over-expressing ubiquitinated form of PXR with TNFα and rifampicin selectively represses PXR-mediated gene activation, but has no effect on NF-κB activity. These phenomena appear to occur in a promoter-selective fashion, providing a possible explanation for how inflammation selectively represses hepatic DMEs that are regulated through the PXR-mediated gene activation program.

Patients experiencing acute or systemic inflammation are recognized as being at increased risk for ADRs. Elucidating the precise molecular basis of the interaction between inflammation and drug metabolism will contribute to the development of

novel pharmaceutical strategies that will improve clinical handling and prevention of disease-drug interactions.

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# Chapter 6: Pregnane X Receptor is Sumoylated to Repress the Inflammatory Response

### *6.1 Introduction*

It has been known for 40 years that treatment with the antibiotic rifampicin (Rif), the prototypical activator of the nuclear receptor (NR) protein pregnane X receptor (PXR; NR1I2), tends to suppress humoral and cellular immunological function in liver cells in patients [1]. This phenomenon has clinical significance, especially in HIV-infected patients presenting with comorbid and highly drug-resistant strains of tuberculosis who are being treated with Rif where a compromised immune response is potentially lethal. An improved understanding of the molecular basis of reduced immune function in Rif-treated patients could lead to the development of new therapeutic strategies to combat inflammatory liver diseases. Because PXR is a molecular target of Rif, we hypothesized that the PXR protein is targeted by the inflammatory signaling pathway in some manner so as to compromise the ability of Rif-treated hepatocytes to mount an immunological response to infection and inflammation.

Several reports indicate that key members of the NR superfamily are sumoylated to repress the inflammatory responses in various tissue types. It is noteworthy that Pascual et al., [2] presented a model for repression in mouse macrophages in which ligand-dependent sumoylation of peroxisome proliferator-activated receptor γ results in its recruitment to the promoters of several inflammatory-response genes where it inhibits transcription by preventing clearance of multiprotein corepressor complexes.

Other evidence indicates that ligand-mediated sumoylation of liver X receptor NR proteins plays a critical role in transrepression of inflammatory response genes in cultured brain astrocytes [3].

PXR is highly expressed in liver and is the molecular target of numerous clinically prescribed drugs, drug metabolites, and active ingredients in several widely used herbal remedies [4-7]. Activation of hepatic PXR by these compounds represents the molecular basis of an adaptive response that protects hepatocytes from toxic insult, and at the same time, produces potentially life-threatening drug–drug, herb–drug, and food–drug interactions in patients on combination therapy.

Although much is known regarding the identity of ligands and target genes for PXR, relatively little is known regarding the molecular interface of signal transduction pathways with this important hepatic transcription factor. The PXR protein has recently been shown to be the target of several signal transduction cascades that modulate its phosphorylation status and transcriptional activity [8-10]. A study indicates a significant increase in liver-enriched transcription factor cross-talk in patients with severe liver disease, suggesting that an elevation in the coordinate regulation of hepatic gene expression occurs during the inflammatory response [11]. Two reports have described mutually repressive and negative cross-talk between the PXR and NF-κB signaling pathways [12, 13]. It therefore seems likely that coordinate regulation of genes involved in both inflammation and xenobiotic metabolism occurs as part of a widespread response to the infection and inflammatory responses, although the molecular basis for these phenomena is not fully known.

Species-specific effects are often observed when examining signal transduction pathways and activating ligands of PXR [9, 14]. It is therefore important to examine PXR function in several cell models where possible. Here, we use immortalized cell lines, transgenic "humanized" PXR mice, and primary cultures of mouse and human hepatocytes to show that sumoylation of the PXR protein represents the molecular basis of the diminished inflammatory response observed across species. Our data support the idea that tumor necrosis factor α (TNFα) signaling in hepatocytes produces increased sumoylation of the liganded PXR protein by incorporation of SUMO3 chains. We show here that the sumoylated form of the PXR protein represses NF-κB target gene expression, but has little effect on CYP3A gene expression in reporter gene assays. These data provide a plausible molecular explanation for how the PXR NR protein can be converted from a positive regulator of drug-handling genes to a promoter-specific repressor of NF-κB target genes and the hepatic inflammatory response during therapy with Rif.

### *6.2 Materials and Methods*

***In Vitro Sumoylation Assay.*** Each sumoylation reaction (Enzo Life Sciences Inc., Farmingdale, NY) contained 1 μM recombinant purified PXR (PanVera Corp., Madison, WI) or RanGap1 in total 20-μl volume in the presence or absence of $Mg^{2+}$-ATP. The assay components 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-SUMO1 or anti-SUMO2/3 antibodies (Enzo Life Sciences Inc.). The membrane was stripped and reprobed by using anti-PXR H-11 monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

***Cell-Based Sumoylation Assays.*** Plasmids pcDNA3-6His-SUMO1, pcDNA3-6His-SUMO2, and pcDNA3-6His-SUMO3 were kind gifts from Dr. Ronald T. Hay (University of Dundee, Dundee, United Kingdom). Plasmids p3258 (pCMV-hUBC9) and p3259 (pCMV-hUBC9 C93S) were obtained from Addgene (Cambridge, MA). HeLa cells were maintained in Dulbecco's modified Eagle's medium supplemented with 2 mM l-glutamine, 100 U/ml penicillin/streptomycin, and 10% fetal calf serum. The cell-based sumoylation assay was carried out as described with minor modifications [15]. For transfection assays, HeLa 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 3000g for 15 min. The cleared cell lysates were mixed with 25 μl of $Ni^{2+}$-linked agarose (QIAGEN, Valencia, CA) 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 1000 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 and anti-SUMO2/3 antibodies.

***Transient Transfection and Reporter Gene Analysis.*** The XREM-LUC and NF-κB-LUC reporter gene assays were performed as described previously [5]. In brief, Hela cells were plated in 96-well plates at a density of 7000 cells per well. Cells were transfected by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To measure NF-κB activation, cells were transfected with CMV-β-galactosidase (20 ng), NF-κB-LUC reporter gene (20 ng), pSG5-hPXR (10 ng), and pcDNA3-His-SUMO3 (10 ng). Various amounts of pBluescript were added to wells to achieve 110 ng of total DNA per well. The PXR transactivation assays were performed with CV-1 cells. In brief, cells were transiently transfected with CMV-β-galactosidase (20 ng), XREM-LUC (20 ng), and pSG5-hPXR (5 ng), and pBluescript was added to achieve 110 ng of total DNA per well. Twenty-four hours after transfection cells were treated with either vehicle (0.1% dimethyl sulfoxide) or drug (10 μM Rif) for an additional 24 h. Treatment of cells with TNFα was accomplished by using 10 ng/ml TNFα. Luciferase activities were determined by

using a standard luciferase assay system (Promega, Madison, WI). β-Galactosidase activities were determined by o -nitrophenyl-β-d-galactopyranoside assay, and plates were read at 420 nm.

***Hepatocyte Culture and Treatment.*** Hepatocytes were isolated from congenic (C57BL6) wild-type, PXR knockout, or humanized PXR transgenic mice using a standard collagenase perfusion method as described previously [5, 16]. Hepatocytes were plated in collagen-coated six-well plates at a density of 8 × 105 live cells/well. Primary cultures of human hepatocytes were purchased (Invitrogen). Forty-eight hours after plating, hepatocytes were treated with vehicle, 10 μM Rif, 10 μM pregnenalone 16α-carbonitrile (PCN), 10 ng/ml TNFα, or 25 μM N-(benzyloxycarbonyl)leucinylleucinylleucinal Z-Leu-Leu-Leu-al (MG132) for 24 h.

***Immunoprecipitation of Human PXR Protein.*** After drug treatment, cells were lysed by sonication in a buffer composed of 50 mm Tris-HCl, pH 7.4, 150 mm NaCl, 1 mm EDTA, 1% Triton X-100, and 1 × protease inhibitors (Thermo Fisher Scientific, Waltham, MA). Cell lysates were precleared with 20 μl of immobilized protein A (Repligen, Waltham, MA). Immunoprecipitation of the human PXR protein was accomplished by using a custom polyclonal antibody directed against the human PXR ligand-binding domain. Free immune complexes were captured with immobilized protein A and washed three times with lysis buffer. After SDS-PAGE, the protein was transferred to polyvinylidene difluoride membrane (Millipore Bioscience Research Reagents, Temecula, CA) that was probed with a monoclonal anti-PXR antibody and a rabbit monoclonal anti-SUMO2/3 antibody (Cell Signaling Technology, Danvers,

MA). Immunodetection was performed by using the Pierce ECL Western blotting substrate or SuperSignal west femto maximum sensitivity substrate (Thermo Fisher Scientific) according to the protocol provided by the manufacturer.

***RNA Isolation and Real-Time Quantitative-Polymerase Chain Reaction Analysis.*** Total RNA was isolated from mouse liver or cell culture by using the commercially available reagent TRIzol (Invitrogen), according to the manufacturer's directions. After DNase I treatment, 1 μg of RNA was reverse-transcribed by using random primers following the manufacturer's instruction (Promega). Equal amounts of cDNA were used in real-time quantitative polymerase chain reactions (RT-QPCRs). Reactions included 1 × SYBR Green (Lonza Rockland, Inc., Rockland, ME) and 300 nM primers specific for each gene. The primer sets were designed by using the Primer3 program (http://frodo.wi.mit.edu). The sequences (5′ to 3′) for the primers were as follows: 18S, forward primer 5′-AGTCCCTGCCCTTTGTACACA-3′, reverse primer 5′-CGATCCGAGGGCCTCACTA-3′; Cyp3a11, forward primer 5′-CAAGGAGATGTTCCCTGTCA-3′, reverse primer 5′-CCACGTTCACTCCA AATGAT-3′; and IL-1β, forward primer 5′-TTCCAGGATGAGGACATGAG-3′, reverse primer 5′-TTCTGTCCATTGAGGTGGAG-3′. Cycling conditions were 95 ℃ for 2 min followed by 45 cycles of 95 ℃ for 15 s, 60 ℃ for 15 s, and 68 ℃ for 15 s using the Cepheid (Sunnyvale, CA) Smart Cycler system. The fold induction was calculated as described previously [17].

### *6.3 Results*

***The Effect of the PXR Protein on Expression of Inflammatory Cytokines in Liver.*** The NF-κB transcription factor is a key regulator of the inflammatory response in various disease states and tissues [18, 19]. We previously developed a genetically engineered line of mice that lack the Pxr gene (PXR-KO) [4]. We isolated total RNA from livers of congenic wild-type and PXR-KO mice and examined the relative expression levels of several known NF-κB target genes. Analysis of the expression levels of genes encoding TNFα, IL-6, IL-1α, and IL-1β using RT-QPCR showed that the expression levels of these inflammatory cytokines were significantly increased in the livers of PXR-KO mice (Figure 6-1). In particular, the IL-1β gene expression level was dramatically increased (~20-fold) in the livers isolated from the PXR-KO mice compared with wild-type mice. These results reveal an active role for PXR in repressing expression of genes that encode key inflammatory cytokine in liver. These data provide supporting evidence for establishing the existence of transcription factor cross-talk between the PXR and NF-κB in liver.

We next examined IL-1β gene expression levels after treatment of primary cultures of hepatocytes isolated from either wild-type or PXR-KO mice with PCN, TNFα, or both PCN and TNFα. Hepatocytes were treated with the prototypical rodent PXR activator PCN for 48 h, and then with TNFα for an additional 12 h. Treatment of wild-type hepatocytes with PCN alone produced significant repression of IL-1β mRNA expression (Figure 6-2A). In contrast, treatment of PXR-KO hepatocytes with PCN did not repress expression of IL-1β. As expected, the expression level of IL-1β

**Figure 6-1**

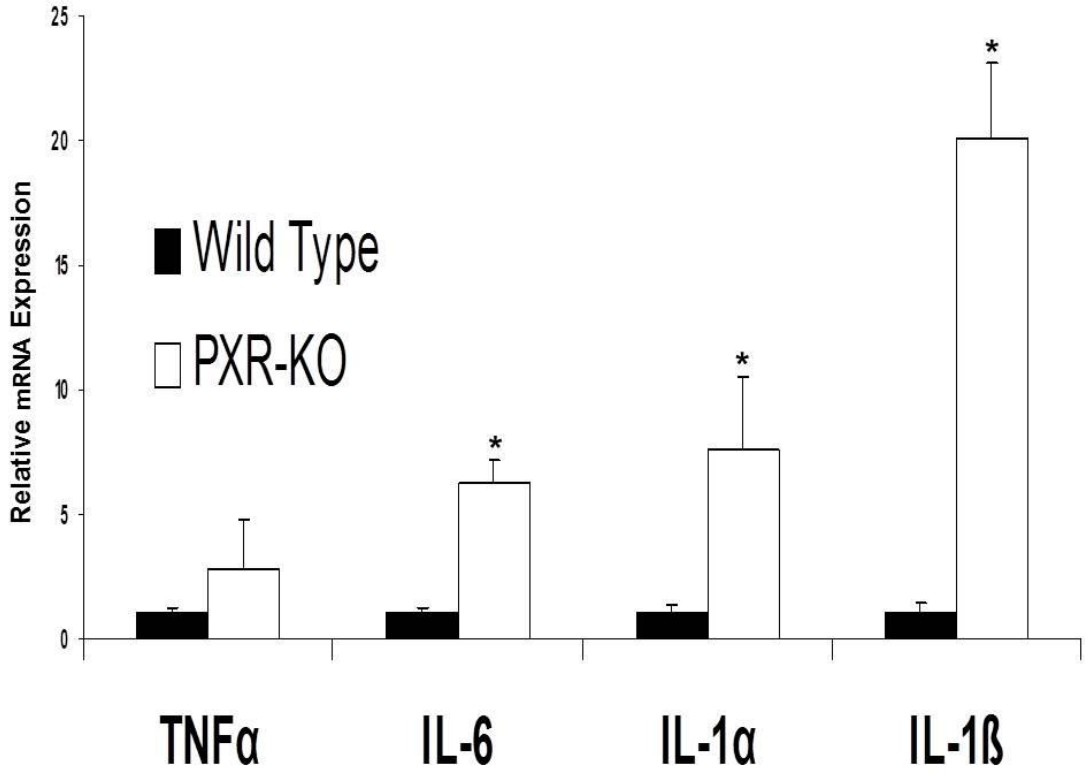


**Figure 6-1. Ablation of PXR from Mice Increases Expression of Inflammatory Cytokines in Liver.** Livers were isolated from wild-type and PXR-KO mice (n = 3), and total RNA was collected. The relative expression levels of TNFα, IL-6, IL-1α, and IL-1β were determined by RT-QPCR. Data are expressed as relative expression in PXR-KO mice compared with that observed in wild-type mice and are normalized to 18S. ∗, $p < 0.01$.

was elevated in PXR-KO hepatocytes (Figure 6-2B). TNFα treatment produced a significant increase in the level of IL-1β mRNA that was effectively repressed by cotreatment of wild-type hepatocytes with TNFα and PCN. PCN-mediated repression of TNFα-inducible IL-1β expression was completely absent from hepatocytes isolated from PXR-KO mice. Moreover, the fold increase of IL-1β mRNA expression produced by TNFα was dramatically elevated in PXR-KO hepatocytes compared with wild-type hepatocytes (Figure 6-2, note the scales). These data indicate an active and suppressive role for liganded PXR in regulating the expression of IL-1β mRNA in response to TNFα.

***The PXR Protein Is SUMOylated In Vitro.*** A bioinformatic approach was used to scan the amino acid sequence of PXR for the presence of a consensus sumoylation sequence (Figure 6-3A). Using this strategy we identified four potential sites of sumoylation within human PXR. We next used in vitro methods to determine the extent to which purified recombinant human PXR serves as a substrate in the SUMO-conjugation pathway. We incubated His-tagged purified recombinant human PXR in vitro together with purified E1, E2, SUMO1, SUMO2, or SUMO3 proteins in the presence and absence of the required magnesium and ATP cofactors. The known SUMO1 substrate RanGap was used as a positive control to ensure the integrity of the in vitro conjugation system. The extent of sumoylation after the incubation was determined by SDS-PAGE and Western blot analysis with commercially available antibodies that recognize either SUMO1 or SUMO2/3 proteins (Figure 6-3B, left) or

**Figure 6-2A**

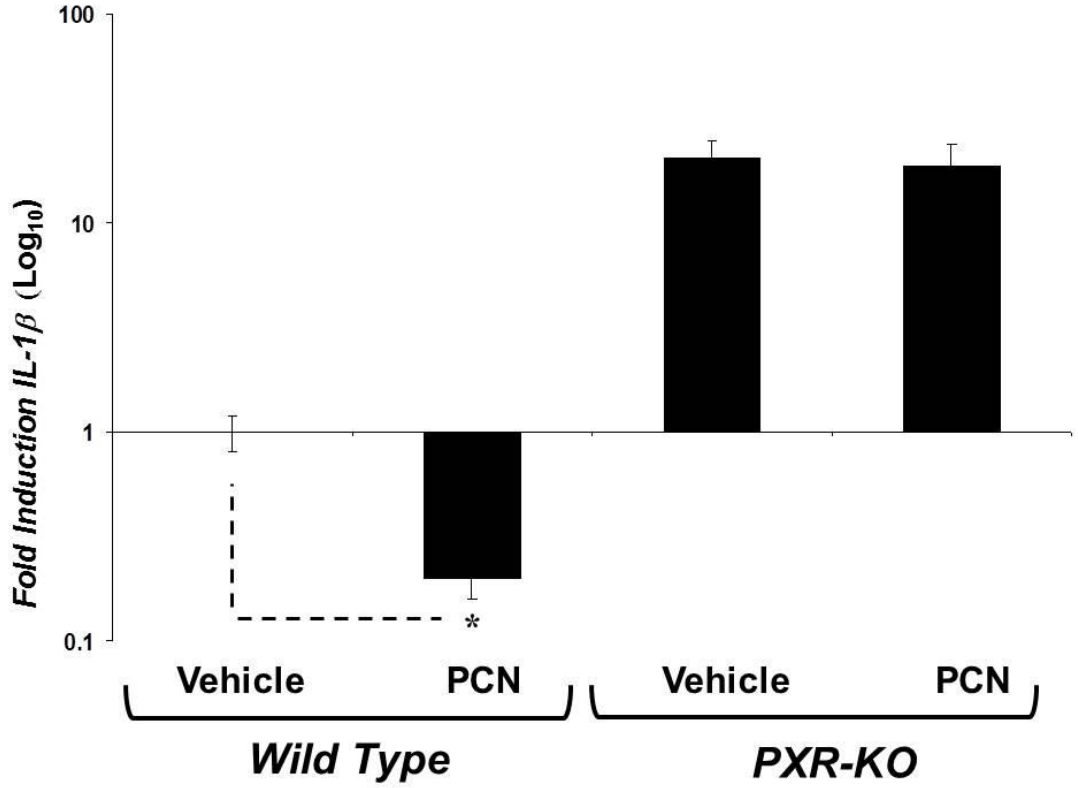
100
10
1
0.1
Fold Induction IL-1β (Log10)
*
Vehicle
PCN
Vehicle
PCN
Wild Type
PXR-KO

**Figure 6-2B**

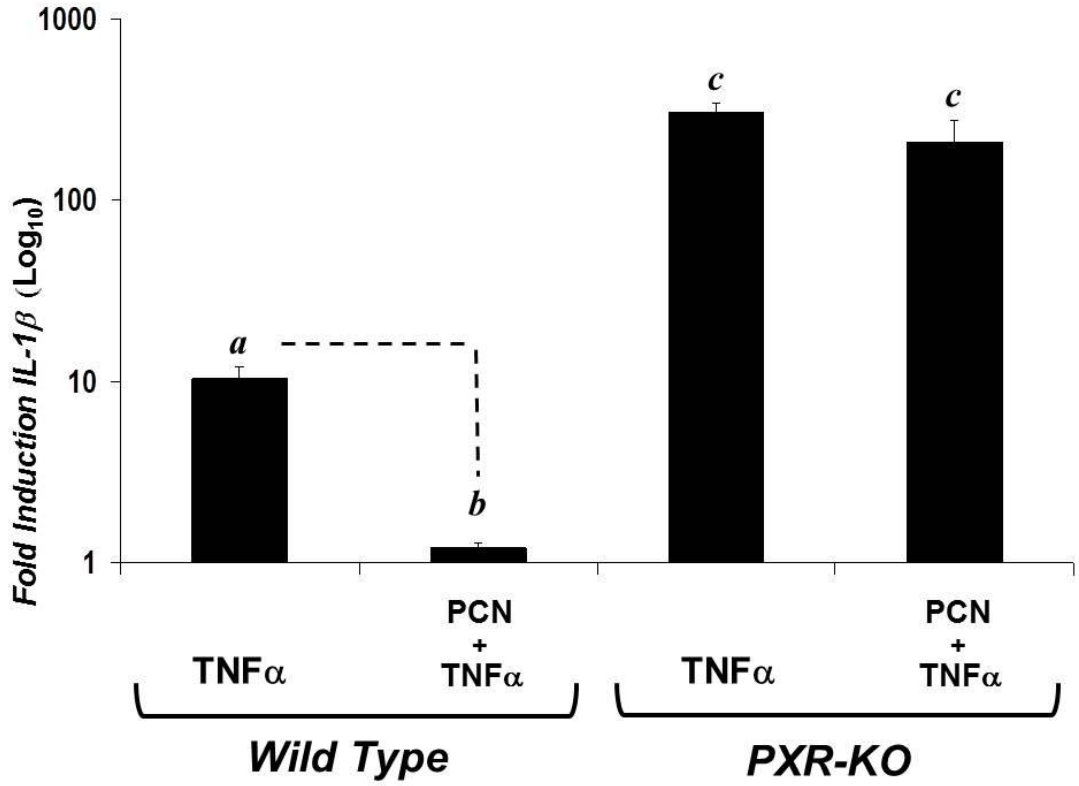


**Figure 6-2. Cotreatment of Wild-type Hepatocytes with PCN and TNFα Represses Expression of IL-1β in Liver, but not in Hepatocytes from PXR-KO Mice.** Primary hepatocytes were isolated from wild-type (A) and PXR-KO (B) mice. Cells were cultured for 24 h and then treated with 10 μM PCN for 48 h before the addition of TNFα (10 ng/ml) for an additional 12 h. Total RNA was collected, and the relative expression level of IL-1β mRNA was determined by RT-QPCR. Data are expressed as relative expression (Log10 scale) compared with that observed in vehicle-treated wild-type cells and are normalized to 18S. In A, ∗, $p < 0.01$. In B, letters different from each other are significantly different ($p < 0.01$).

**Figure 6-3A**

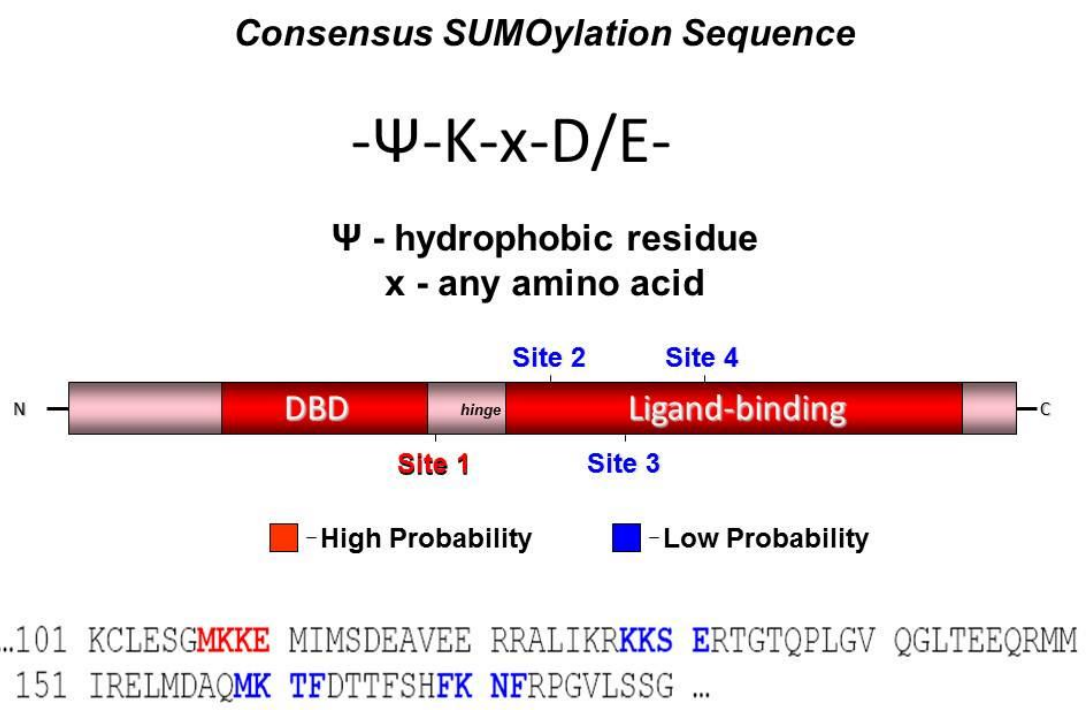


**Figure 6-3A. Analysis of Consensus Sumoylation Sites in the Human PXR Protein.** The human PXR protein was analyzed for the presence of the consensus sumoylation sequence as defined by an online SUMOPlot server (http://www.abgent.com/tools/SUMOplot). This type of bioinformatic analysis identifies four 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 6-3B**

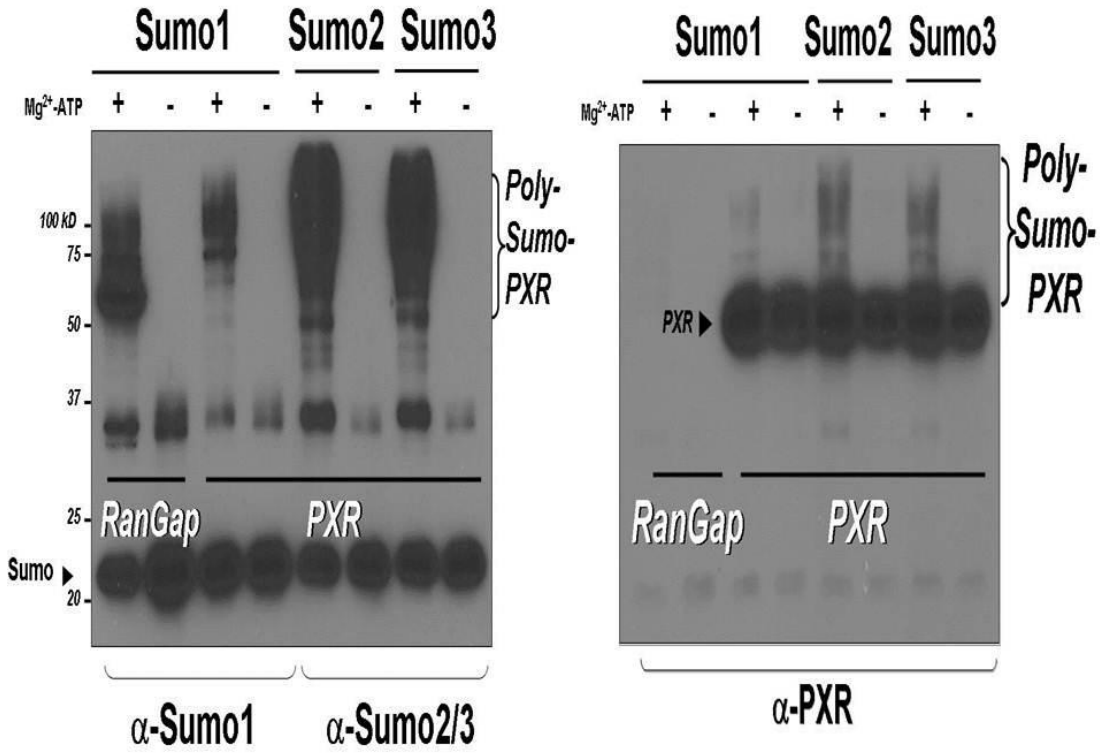


**Figure 6-3B. In Vitro Sumoylation of Human PXR Protein.** The human PXR protein was used as a test substrate for SUMO1, SUMO2, and SUMO3. Left, the protein was detected by Western blot analysis with antibodies that recognize either SUMO1 or SUMO2/3. The RanGap protein was used as a positive control for experimental integrity. Right, the same blot was stripped and reprobed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein.

with antibodies that recognize the human PXR protein (Figure 6-3B, right). This type of analysis reveals that the human PXR protein can serve as an effective substrate for SUMO1, SUMO2, or SUMO3 in the SUMO-conjugation pathway in vitro. Poly-SUMO chains form on PXR when SUMO2 or SUMO3 are used in the reaction. Studies confirm that the Ubc9 enzyme can effectively catalyze the formation of poly-SUMO chains in vitro [20, 21]. The functional significance of the formation of poly-SUMO chains on PXR is currently unknown. Although an in vitro approach is highly suggestive of potential PXR sumoylation, it is also necessary to demonstrate that PXR is sumoylated in cultured cell lines.

***PXR is Preferentially Sumoylated in Cultured Cells by SUMO3.*** We have initiated a series of studies using an overexpression and transfection approach in HeLa, CV-1, and HepG2 cultured cells. For brevity, we will provide the data obtained using HeLa cells; however, the data obtained using either CV-1 or HepG2 cells are identical (data not shown). We used cDNA expression vectors encoding 6×His-tagged SUMO1, SUMO2, and SUMO3 proteins together with an expression vector that encodes the human PXR protein. Cotransfection of HeLa cells with the PXR expression vector together with the 6×His-SUMO1, 6×His-SUMO2, or 6×His-SUMO3 expression vectors allows the rapid and selective purification of sumoylated forms of PXR using nickel-linked agarose and a strong denaturing buffer containing high levels of guanidine-HCl. The SUMO proteases that probably would cleave sumoylated forms of PXR upon cell lysis are rapidly deactivated under these denaturing conditions. Using this experimental approach we detected sumoylated

PXR using a Western blot with the α-PXR monoclonal antibody (Figure 6-4A, lane 6). Moreover, addition of an expression vector encoding the Ubc9 E2 SUMO ligase induced the formation of poly-SUMO3 chains on PXR, which was present at a lower level in cells expressing only endogenous Ubc9 (Figure 6-4A, lanes 6 and 7). It is noteworthy that addition of an expression vector encoding the dominant-negative Ubc9 (C93S) dramatically reduced poly-SUMO chain formation (Figure 6-4A, lane 8). It is interesting to note that the human PXR protein was preferentially modified in cells by SUMO3.

The use of dominant-negative Ubc9 (C93S) can further determine the specificity of SUMO3 chain formation on PXR. If SUMO-3 chain formation on PXR is occurring, then increasing amounts of dominant negative Ubc9 (C93S) expression will inhibit PXR sumoylation in a dose-dependent manner. Indeed, expression of increasing amounts of dominant-negative Ubc9 (C93S) inhibits sumoylation of PXR by SUMO3 in cultured cells (Figure 6-4B, left, lanes 3–5). It is noteworthy that the same blot was stripped and reprobed with the α-SUMO2/3 antibody and produced confirmatory results that reveal a decrease in SUMO3 immunoreactivity (Figure 6-4B, right, lanes 3–5). These data demonstrate specific conjugation of poly-SUMO3 chains to the human PXR protein in cultured cells. Because forced overexpression of PXR together with SUMO3 and Ubc9 could potentially lead to the production of experimental artifacts, we next sought to examine sumoylation of endogenous PXR in primary cultures of hepatocytes.

**Figure 6-4A**

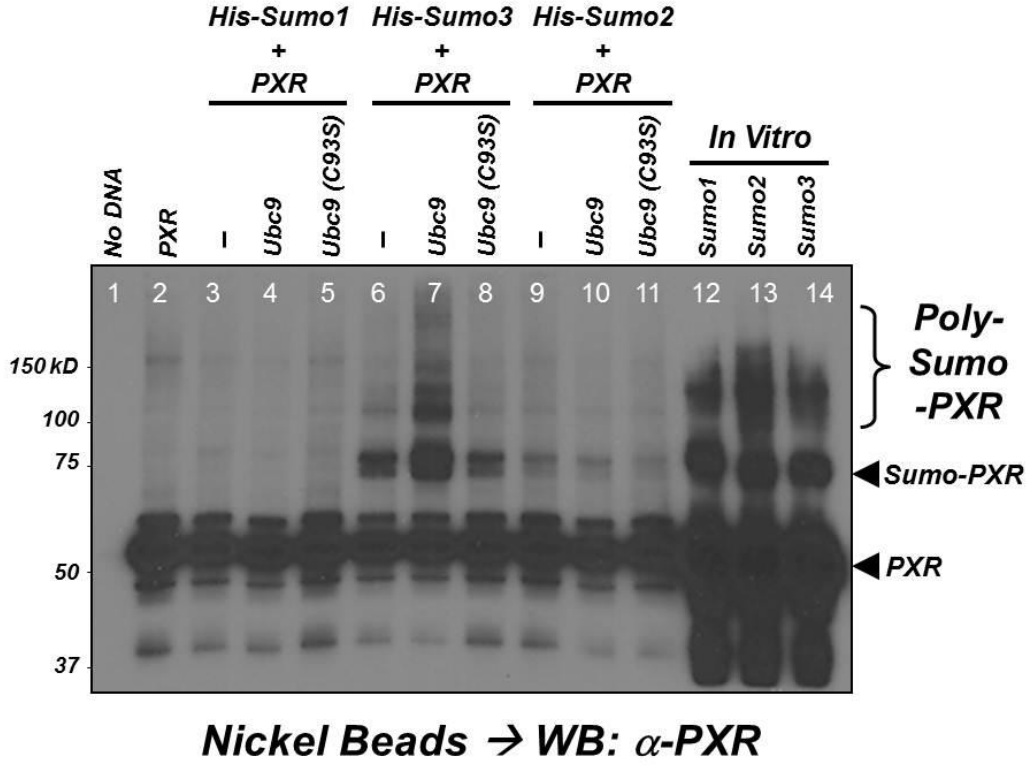


**Figure 6-4A. Detection of Sumoylated Human PXR Protein in HeLa Cells.** The human PXR protein was coexpressed in HeLa cells together with either His-tagged SUMO1, SUMO2, and SUMO3 proteins. Cells were lysed using denaturing buffer containing guanidinium hydrochloride to inactivate de-sumoylation enzymes. Sumoylated proteins were purified by using nickel-linked agarose beads. The blot was probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein.

**Figure 6-4B**

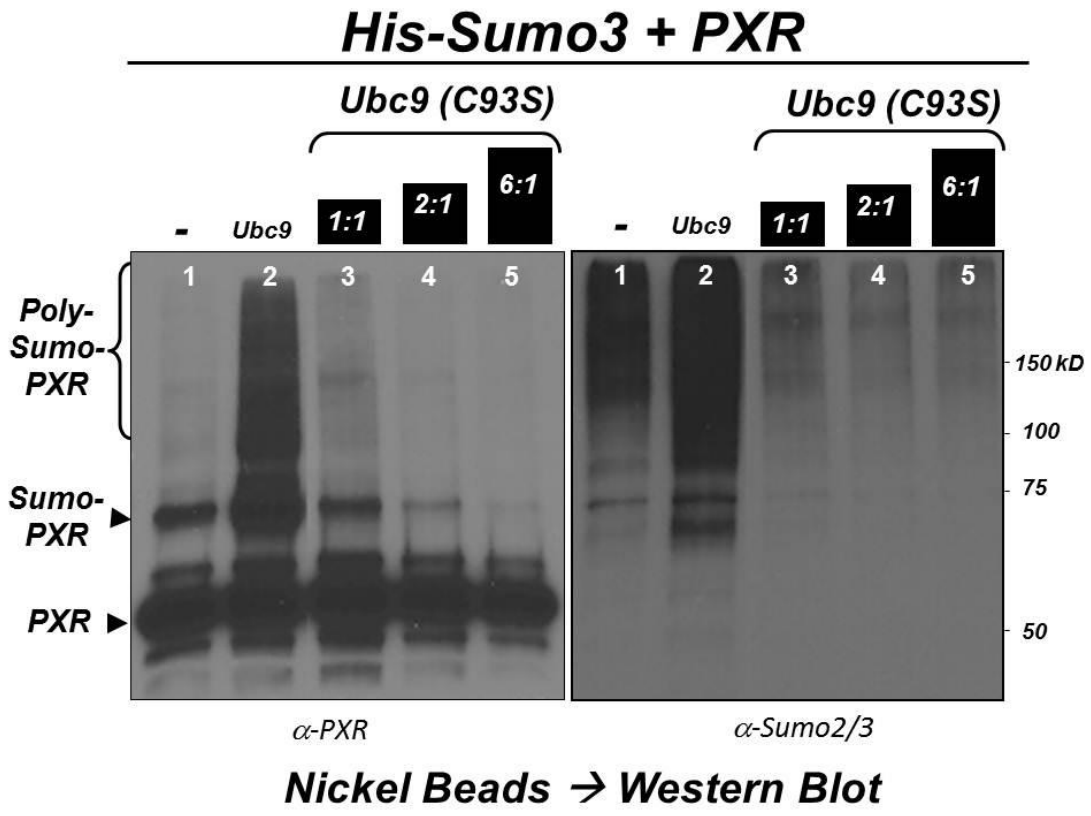


**Figure 6-4B. Dominant-negative Ubc9 (C93S) Protein Inhibits Sumoylation of Human PXR in a Dose-dependent Manner.** The human PXR protein was coexpressed in HeLa cells together with 6×His-tagged SUMO3 and increasing amounts of dominant-negative Ubc9 (C93S). Sumoylated protein was purified using nickel-linked agarose beads. Left, the blot was probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein. Right, the blot was stripped and reprobed with an antibody that recognizes SUMO2/3.

***The Endogenous PXR Protein is SUMOylated in Response to TNFα in Cultured Hepatocytes.*** Although a transfection-based approach using immortalized cell lines is a valid strategy for detecting SUMO-modified PXR protein, an important next step is the use of primary cultures of hepatocytes. NR proteins are degraded by the proteasome (reviewed in [22]). Because of the expected low stoichiometry of PXR sumoylation, we treated cultured human hepatocytes with MG132, a potent inhibitor of proteasomal degradation. We subsequently performed immunoprecipitation of cell extracts using a well characterized custom anti-human PXR polyclonal antibody [9]. The rationale for this experimental approach is that inhibition of proteasome-mediated protein degradation would increase the likelihood of successful detection of the sumoylated form of PXR protein. It is noteworthy that this experimental approach also enabled us to perform important control experiments using cross-detection with both anti-PXR and anti-SUMO2/3 antibodies. Indeed, Western blot analysis using an anti-PXR monoclonal antibody performed on PXR-immuno-enriched cell extracts detected the enrichment of a band of the expected size of sumoylated PXR protein (75 kDa) (Figure 6-5 A, middle). When the blot was stripped and reprobed with the anti-SUMO2/3 antibodies, we detected a band of the identical size that was enriched after treatment with MG132 (Figure 6-5A, bottom), thereby further validating our antibody-based experimental approach.

We have created a novel line of "humanized" PXR transgenic mice in our laboratory. This line of mice harbors the FLAG-tagged human PXR transgene (hPXRtg) whose expression is under the control of the transthyretin promoter.

Crossing this strain of transgenic mice with the PXR-KO mice has created a novel line of humanized PXR transgenic mice that express the FLAG-tagged version of the protein exclusively in liver [16]. Primary cultures of both wild-type and transgenic humanized PXR hepatocytes were treated with vehicle, rifampicin, TNFα, or TNFα plus rifampicin for 24 h. Immunoprecipitation with the anti-hPXR polyclonal antibody followed by Western blot with a monoclonal anti-PXR antibody was performed. The 70-kDa band was increased by treatment with rifampicin and TNFα and cotreatment with rifampicin and TNFα exclusively in the transgenic humanized PXR mice (Figure 6-5B). It has already been established that the PXR-KO mice have elevated levels of TNFα and related inflammatory cytokines [13]. Thus, treatment of humanized PXR mice, which lack expression of murine PXR in small intestine, with rifampicin alone increased sumoylation in this model, probably because of the presence of increased levels of inflammatory cytokines such as TNFα or IL-1β. It is interesting to note that our custom anti-hPXR antibody directed against the ligand-binding domain of human PXR does not capture the murine PXR protein when used for immunoprecipitation from extracts isolated from wild-type mice. These data indicate that the human PXR protein is sumoylated in response to TNFα treatment when expressed in mouse hepatocytes. Taken together, the data presented in Figure 6-5, A and B reveal that our antibody-based experimental approach successfully detects accumulation of sumoylated PXR protein.

Our working hypothesis is that inflammatory signaling pathways increase sumoylation of liganded PXR protein to repress NF-κB transcriptional activity in

human hepatocytes. Primary cultures of human hepatocytes were therefore treated with vehicle, Rif, TNFα, or both Rif and TNFα for 48 h. Total cell extract was subjected to standard preclearing methods and subsequent immunoprecipitation techniques using the anti-hPXR polyclonal antibody. As before, equal loading was determined by using an aliquot of whole-cell lysate and Western blotting to detect β-actin (Figure 6-5C, top). Subsequent Western blot analysis with a monoclonal α-SUMO2/3 rabbit antibody detected a band at the expected size of sumoylated PXR protein (~70 kDa) (Figure 6-5C, bottom). Treatment of cells with TNFα alone or TNFα together with Rif produced an increased level of sumoylated PXR. These results indicate that TNFα produces increased levels of detectable SUMOylated PXR protein in human hepatocytes.

***SUMOylation of PXR Represses TNFα-Inducible NF-κB Reporter Gene Activity.*** Our experiments using cultures of hepatocytes provide compelling evidence that TNFα signaling increases levels of SUMO-modified PXR protein. We next sought to determine the functional role of sumoylated PXR protein using a transient transfection approach together with an NF-κB-luciferase reporter gene strategy. This reporter gene contains an NF-κB-response element (-TGGGGACTTTCCGC-) multimerized five times. Previous studies in our laboratory using transient transfection and PXR in 96-well reporter gene assays were performed with CV-1 cells [6, 8]. It is noteworthy that treatment of cultured CV-1 cells with TNFα produced an approximate 10-fold increase in NF-κB reporter gene activity, whereas treatment with Rif did not have any effect on NF-κB reporter gene alone or in combination with

**Figure 6-5A**

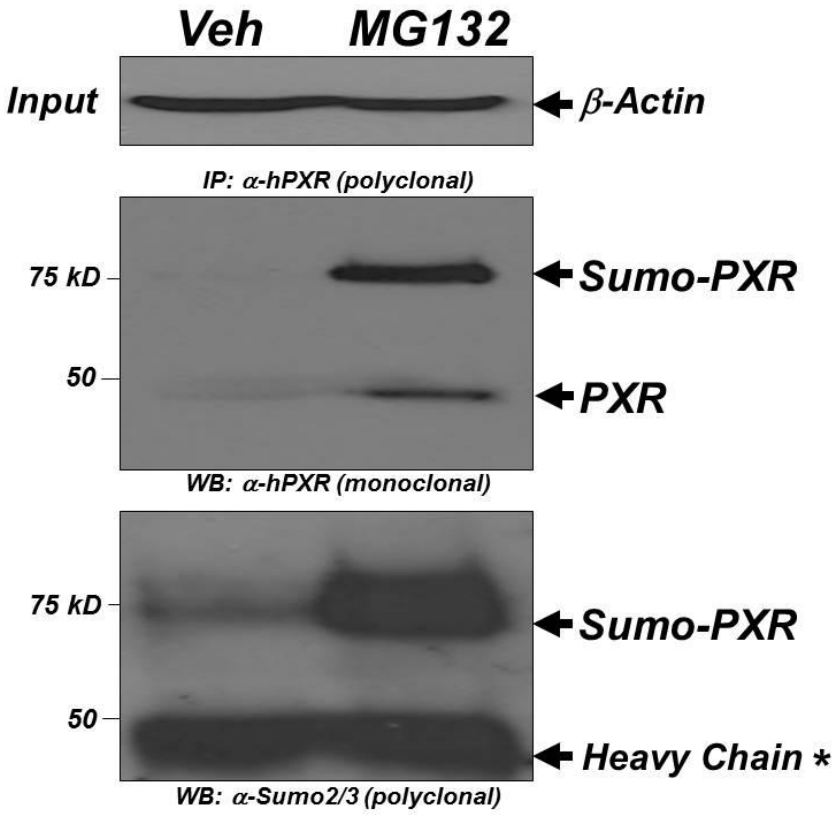
Veh
MG132
Input
β-Actin
IP: α-hPXR (polyclonal)
75 kD
50
Sumo-PXR
PXR
WB: α-hPXR (monoclonal)
75 kD
50
Sumo-PXR
Heavy Chain *
WB: α-Sumo2/3 (polyclonal)

**Figure 6-5B**

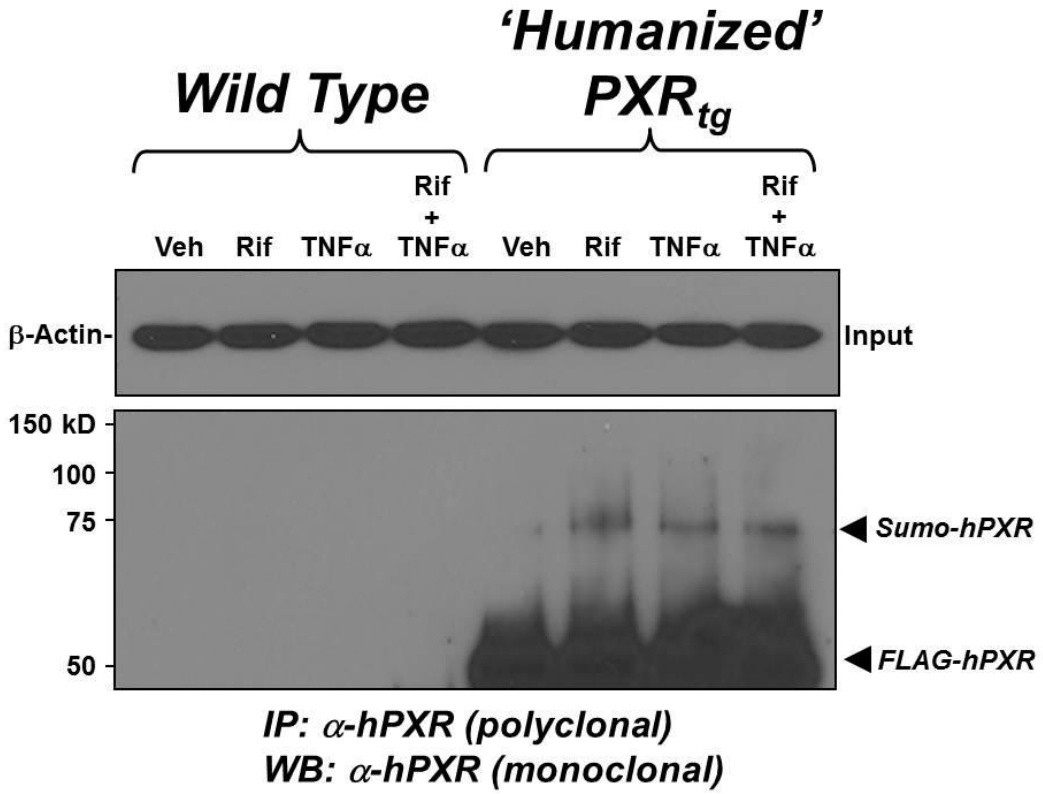
'Humanized'
Wild Type
PXR$_{tg}$
Rif + TNFα
Veh
Rif
TNFα
Veh
Rif
TNFα
β-Actin-
Input
150 kD
100
75
50
Sumo-hPXR
FLAG-hPXR
IP: α-hPXR (polyclonal)
WB: α-hPXR (monoclonal)

**Figure 6-5C**

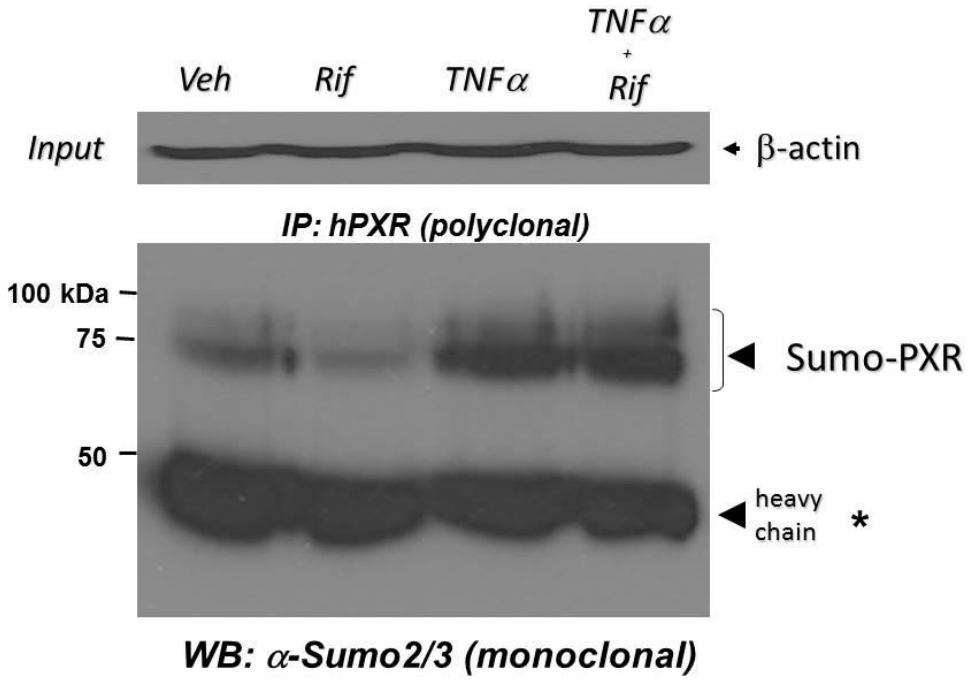


**Figure 6-5. Detection of Sumoylated PXR in Hepatocytes.** Primary cultures of hepatocytes isolated from human donors (A and C) or humanized PXR mice (B) were treated for 48 h with Rif (10 μM), TNFα (10 ng/ml), or TNFα + Rif. Whole-cell protein lysates were subjected to SDS-PAGE and blotted with antibodies against β-actin to ensure equal loading of the subsequent immunoprecipitation experiment (top). Whole-cell lysates were subjected to immunoprecipitation with the polyclonal antibody that recognizes human PXR. Immunoprecipitates were resolved using SDS-PAGE and subjected to Western blot analysis using antibodies that recognize human PXR or SUMO2/3 as indicated. ∗ indicates cross-reaction with the secondary antibody caused by the presence of heavy chain.

TNFα. However, addition of the PXR expression vector (10 ng/well) either alone or in combination with SUMO3 (10 ng/well) and Ubc9 (10 ng/well) effectively repressed TNFα-mediated NF-κB reporter gene activity (Fig. 6 A). Titration of the dominant-negative Ubc9 (C93S) expression vector (5, 10, and 25 ng per well) restored TNFα-mediated increases in NF-κB reporter gene activity in a dose-dependent manner (Figure 6-6B). It is noteworthy that expression of SUMO3 and Ubc9 had no effect on PXR-mediated gene activation when directed toward the PXR response element-controlled luciferase reporter gene (Figure 6-6C).

### *6.4 Discussion*

Several NR proteins play key roles in regulating inflammatory processes. Among these receptors, the glucocorticoid receptor was the first to be characterized as a negative regulator of genes encoding cytokines, adhesion molecules, and inflammatory receptors through interactions with the activator protein-1 transcription factor [21, 23]. A key feature of this repression was that it occurred in the absence of DNA binding and was therefore thought to be mediated through protein–protein interactions. A later study indicated that the molecular basis for the well-known suppression of inflammatory processes by Rif was also mediated through the glucocorticoid receptor [24]. However, additional studies were unable to corroborate this finding [25]. Thus, the molecular basis of Rif-mediated suppression of inflammation has remained obscure. Although two other articles have identified the existence of strong repressive cross-talk between the PXR and NF-κB signaling pathways [12, 13], no well-defined molecular

**Figure 6-6A**

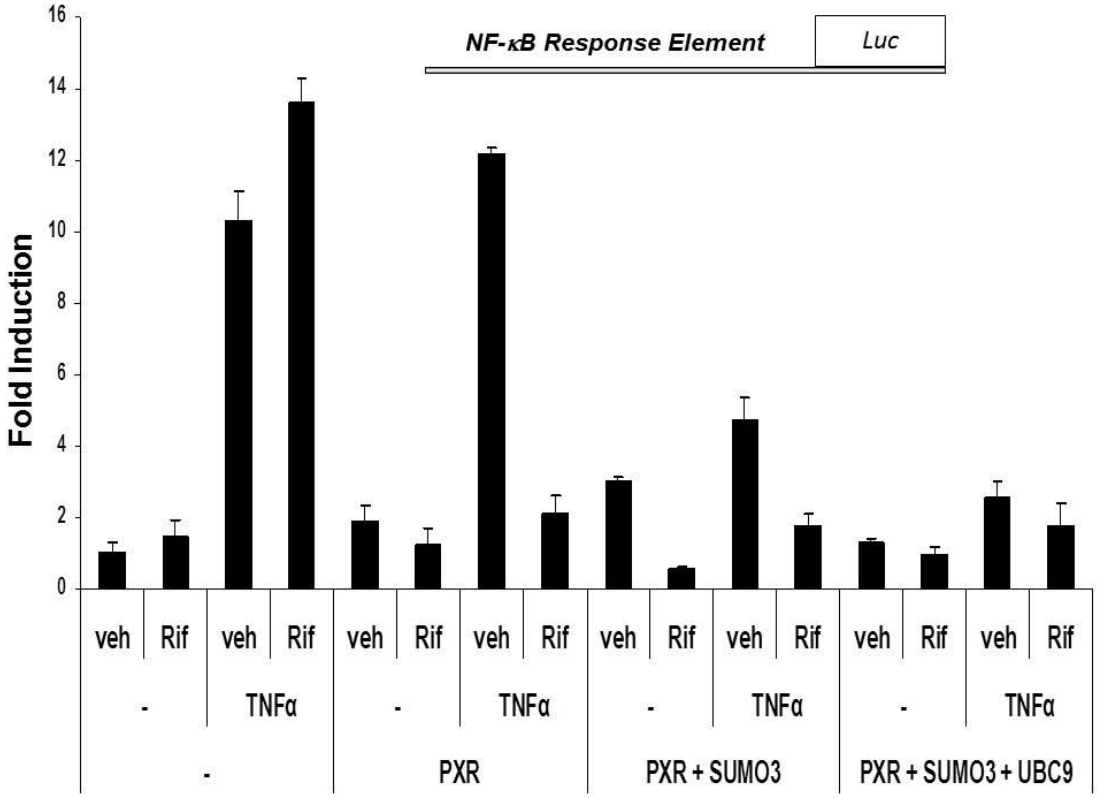


**Figure 6-6. Functional Significance of SUMO3 Modification of the PXR Protein.**

A, CV-1 cells were transfected with an NF-κB-luciferase reporter gene (20 ng/well) in the presence and absence of various combinations of PXR (10 ng/well), SUMO3 (10 ng/well), and Ubc9 (10 ng/well). Luciferase activity was determined by using standard methods, is reported as fold-induction ± S.E.M., and was normalized to β-galactosidase activity.

**Figure 6-6B**

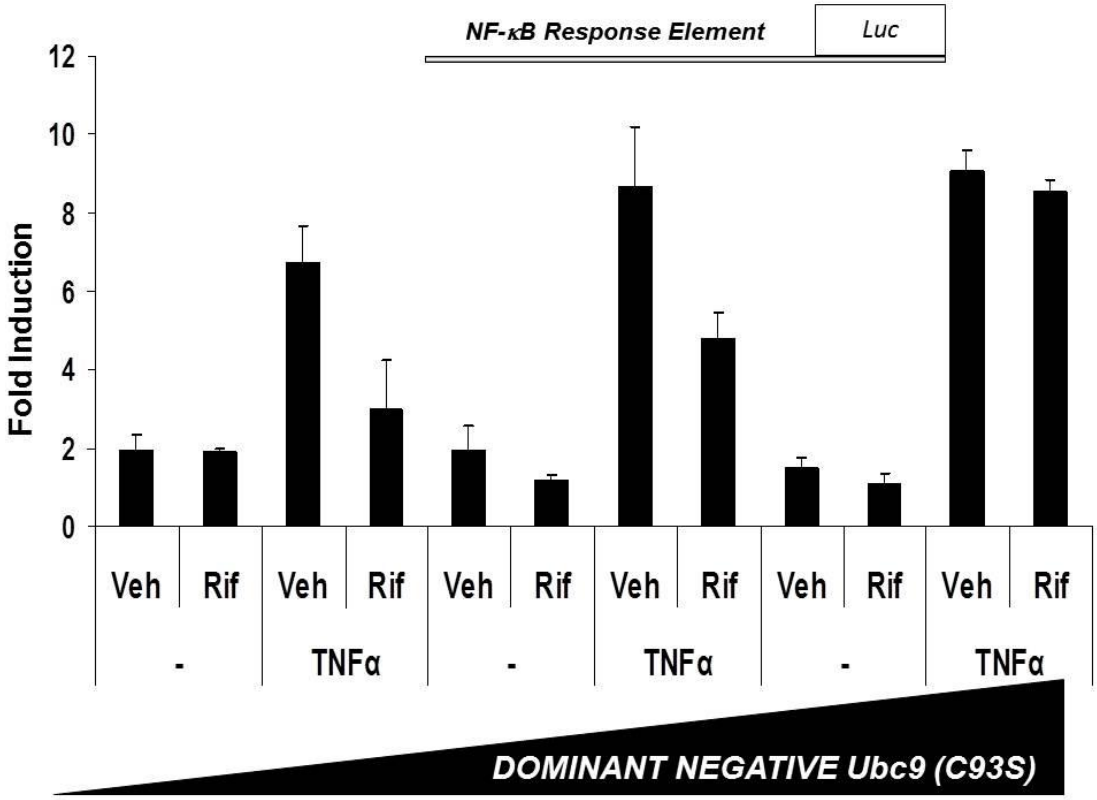


**Figure 6-6. Functional Significance of SUMO3 Modification of the PXR Protein.**

B,CV-1 cells were transfected as in A; however, increasing amounts of dominant negative Ubc9 (C93S) were included (5, 10, and 25 ng/well). Luciferase activity was determined by using standard methods, is reported as fold-induction ± S.E.M., and was normalized to β-galactosidase.

**Figure 6-6C**

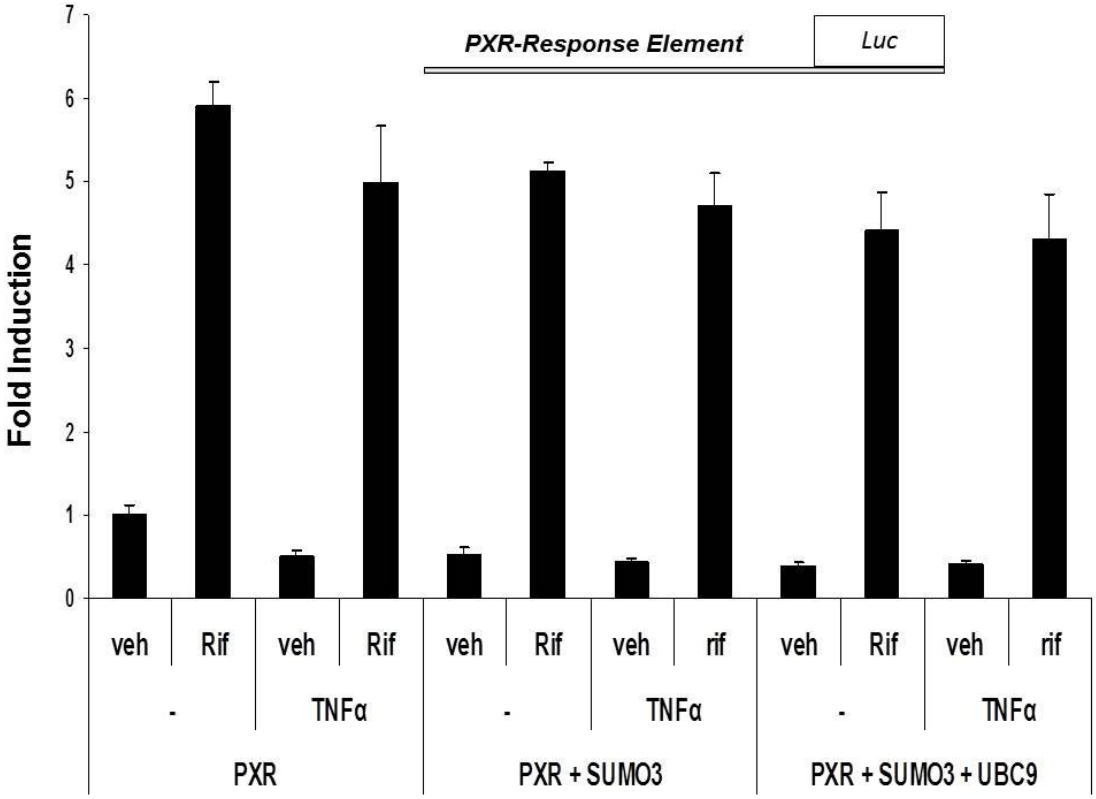


**Figure 6-6. Functional Significance of SUMO3 Modification of the PXR Protein.**

C, CV-1 cells were transfected as in A except a PXR-response element luciferase reporter gene (XREM-LUC) was used. Veh, vehicle.

mechanism for Rif-mediated repression of the inflammatory response in hepatocytes was identified. The data presented here are consistent with a review article that highlights the increased recognition of the counter-regulatory role of several liver- and intestine-enriched NR proteins in entero-hepatic immune responses [26]. The data we present here identify sumoylation of PXR as the likely molecular basis for inhibition of the hepatic immune response in Rif-treated patients. Our data also form the basis of a new molecular paradigm that will seek to exploit the interface between ligand-mediated PXR activation, PXR sumoylation, and inflammatory liver and bowel diseases.

The sumoylation pathway begins with a SUMO-activating enzyme (also called E1), which carries out an ATP-dependent activation of the SUMO C terminus and then transfers the activated SUMO protein to a SUMO-conjugating enzyme (E2 ligase) called Ubc9. Ubc9 is the only known E2 SUMO ligase. In vivo, the SUMO moiety is then transferred from Ubc9 to the substrate with the assistance of one of several E3 SUMO-protein ligases. When this reaction is carried out in vitro, the E3-SUMO ligase is dispensable. The human genome contains three functional genes that encode SUMO proteins called SUMO1, SUMO2, and SUMO3. The three SUMO proteins seem to have different biological functions, but the three-dimensional structures are very similar to each other and also share a high degree of structural similarity to the ubiquitin protein. Moreover, there is a preference among substrates for the different SUMO proteins. Although sumoylation controls a large number of cellular processes, it clearly plays a prominent role in the repression of transcription [27]. In fact, the

consensus sumoylation site was identified as a negative regulatory sequence in a bioinformatics comparison of several transcription factors before it was identified as a site for sumoylation [28]. When either SUMO or the Ubc9 proteins are tethered to DNA through DNA-binding domains such as the GAL4 system, strong transcriptional repression is observed [29, 30]. It is noteworthy that in this context the SUMO-2/3 proteins exhibited greater repression compared with SUMO-1 [29]. Whereas SUMO-1 seems to be conjugated mostly to proteins, the SUMO-2/3 proteins are found primarily in a free form. However, an increase in SUMO-2/3 incorporation into substrates is detected after exposure to several stress conditions, including heat shock [15]. The data presented here provide additional evidence to include xenobiotic stress in playing a role in increased conjugation of SUMO3 to the PXR protein. Conjugation of SUMO3 chains to PXR is therefore likely to be intimately involved in mediating active repression of NF-κB activity in liver cells. If analogous to peroxisome proliferator-activated receptor-mediated repression, a molecular mechanism for this repression could include selective targeting of PXR to NR corepressor/histone deacetylase 3 complexes on inflammatory gene promoters [2]. A working model for this hypothesis is shown in Figure 6-7.

PXR regulates key aspects of drug metabolism and drug transporter activity in several key tissue types, including liver and intestine, and in capillary endothelial cells that comprise the blood-brain barrier [4, 17, 31, 32]. PXR is the molecular target of numerous clinically prescribed drugs, drug metabolites, active ingredients in several

**Figure 6-7**

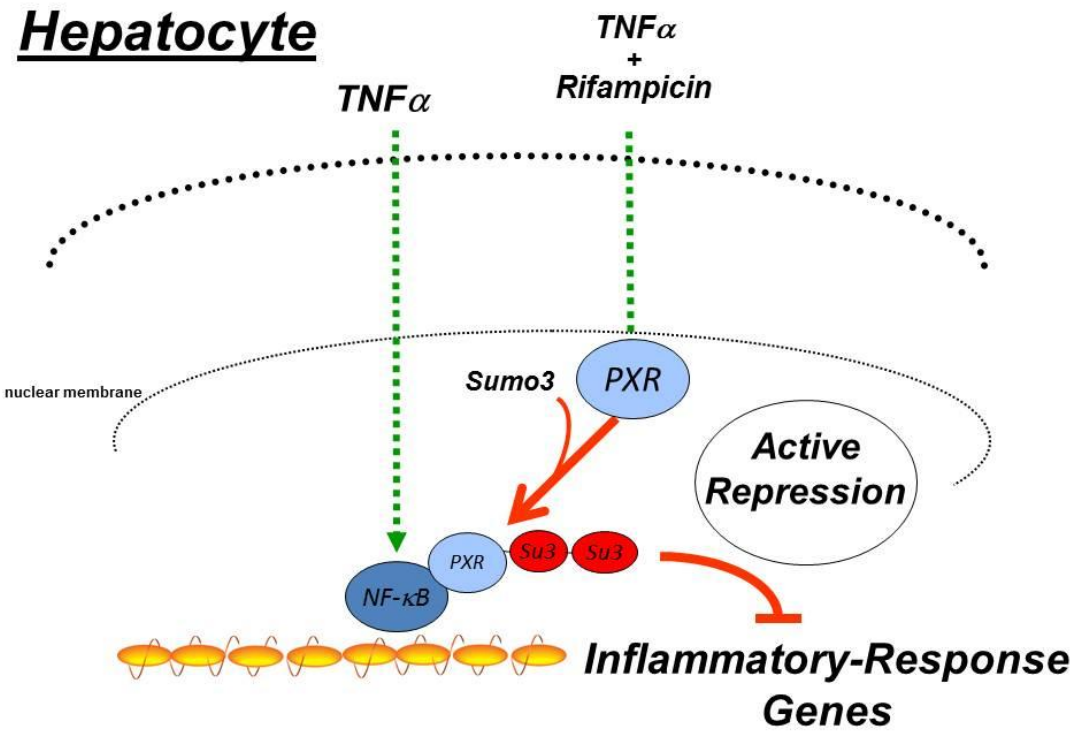


**Figure 6-7. Model of PXR-mediated Repression of Inflammatory Response Pathways.** The TNFα-mediated inflammatory response strongly modulates sumoylation of ligand-bound PXR protein to actively repress the expression of inflammatory response genes.

widely used herbal remedies, and endobiotic compounds [4-6, 16]. Activation of PXR by these compounds represents the molecular basis of an adaptive response that protects cells from toxic insult and at the same time produces potentially life-threatening drug–drug, herb–drug, and food–drug interactions in patients on combination therapy. Previous evidence for PXR involvement in transrepression of the inflammatory response is derived from the PXR-KO mouse model. A study by Teng and Piquette-Miller [33] revealed that PXR-KO mice exhibit significant diminution of endotoxin-mediated suppression of the expression of the Mrp2 gene in liver. Other investigations have revealed that the activation of NF-κB and PXR somehow produces transrepression of the expression of each other's target genes [13]. It is noteworthy that this study showed that the PXR-KO mice exhibit elevated markers of inflammation in the small bowel compared with wild-type mice, including significantly increased expression levels of several key NF-κB target genes, including cyclooxygenase 2, IL-6, TNFα, IL-2, IL-1α, IL-1β, IL-15, and intercellular adhesion molecule 1. Disruption of the molecular interaction between PXR and DNA through increased protein–protein interaction between the p65 subunit of NF-κB and retinoid X receptor has been proposed as the molecular basis for transrepression of the xenobiotic response by inflammatory cytokines [12], although the precise mechanism that gives rise to the selective interaction between these two proteins is not currently known. Several studies indicate that PXR-mediated inhibition of NF-κB is required for antifibrogenic effects and repression of CYP3A4 expression in hepatocytes [34, 35]. Further research will be necessary to elucidate the biochemical details of this response;

however, the data presented here provide a stable platform for launching these important studies.

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# Chapter 7: Expression, Isolation, and Purification of *In Vitro* SUMOylation Components

## *7.1 Introduction*

Most proteins undergo some form of post-translational modification after their synthesis has been completed. Covalent modification of proteins by small ubiquitin-related modifier (SUMO) regulates various cellular functions including protein-protein interaction, sub-cellular localization, regulation of DNA binding, etc [1]. The human genome contains three functional genes that encode SUMO proteins called SUMO1, SUMO2, and SUMO3. The amino acid homology between SUMO2 and SUMO3 is 97%, whereas these two proteins share only 45% amino acid homology with SUMO1 [2].

The mechanism for SUMO conjugation is analogous to that of the ubiquitin conjugation system. All three SUMO proteins are expressed in an immature form, in which they carry a C-terminal stretch of amino acids after a Gly-Gly motif. Before conjugation, nascent SUMO needs to be proteolytically processed to reveal its C-terminal Gly-Gly motif. This is accomplished by SUMO-specific isopeptidases, also known as the SENP SUMO deconjugating enzymes, which remove 4 C-terminal amino acids from SUMO1, 11 amino acids from SUMO2, and 2 amino acids from SUMO3 [3]. Mature SUMO is activated by a SUMO-activating enzyme (also called E1), which carries out an ATP-dependent activation of the SUMO C-terminus. The activated SUMO protein is then transferred to a SUMO-conjugating enzyme (E2 ligase) called Ubc9 [4]. *In vivo*, the SUMO protein is further transferred from Ubc9 to the

lysine residue within the substrate with the assistance of one of several E3 SUMO protein ligases. When this reaction is carried out *in vitro*, the E3-SUMO ligase is dispensable. SUMOylated targets serve as substrates for SENPs, which ensures the reversible and dynamic nature of SUMOylation (Figure 7-1).

Maturation of newly synthesized SUMOs prior to their initial conjugation is accomplished by the action of enzymes called Ubl (ubiquitin-like protein)-specific proteases (Ulp) in yeast and Sentrin-specific proteases (SENP) in mammals [5, 6]. The same group of enzymes is also responsible for SUMO de-conjugation. Ulp/SENPs directly regulate the pools of free, conjugatable SUMO protein and the half-life of conjugated species [7].

SUMO-activating enzyme (E1) is a heterodimer, which consists of two proteins AOS1 (SAE1) and UBA2 (SAE2) [8]. Interestingly, human AOS1 and UBA2 have significant amino acid homology to the N or C-terminal half of E1 enzyme for ubiquitin, respectively. The human E1 heterodimer contains a conserved cysteine residue that functions as an active site that is required for formation of the thioester bond with all members of the SUMO family *in vitro*. Thus, SUMO1, SUMO2, and SUMO3 are activated by the same E1 holo-enzyme [9].

Ubc9, the only identified SUMO E2 enzyme is homologous to E2 ubiquitin conjugating enzyme. It conjugates activated SUMO through a C-terminal isopeptide bond formation to the substrate proteins [10, 11].

Several proteins have been shown to possess SUMO E3 ligase-like properties.

**Figure 7-1**

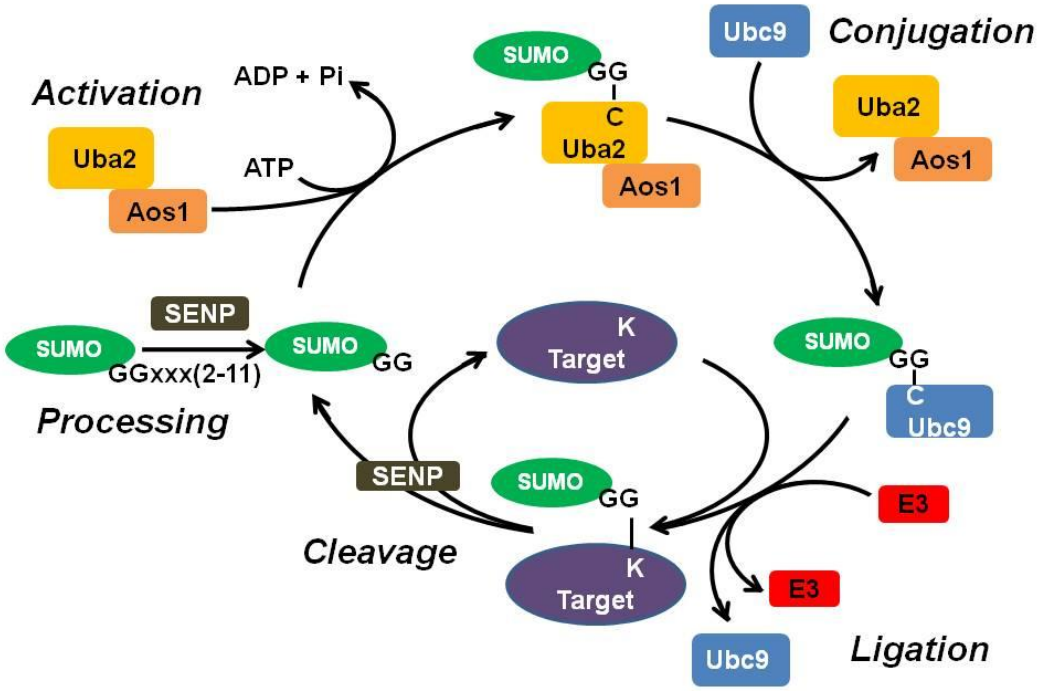


**Figure 7-1. The SUMOylation Pathway.** After the C-terminal processing, SUMO is activated in an APT-dependent reaction by E1 activating enzyme. Subsequently, SUMO is transferred to the E2 conjugating enzyme (Ubc9), and finally the lysine residue within the substrate with the assistance of E3 ligase enzyme. SUMOylation is a reversible process, and SUMO can be cleaved from target proteins by isopeptidases.

The E3 sumo-conjugating enzymes have been categorized into three groups: (1) the PIAS family (protein inhibitor of activated STAT-signal transducer and activator of transcription), (2) RanBP2 (the nuclear pore proteins Ran binding protein 2) , and (3) Pc2 (the polycomb group member) [12-14].

SUMOylation of proteins *in vitro* is a useful tool for research of this post-translational modification. In this chapter, we detail the bacterial expression, isolation, and purification of proteins necessary to perform *in vitro* SUMOylation assays, namely SUMO E1 enzyme (AOS1/UBA2 heterodimer), Ubc9, and SUMO1, SUMO2, and SUMO3. Detailed methods for performing *in vitro* SUMOylation assay by SUMO1, SUMO2, and SUMO3 using RanGap1 as substrate are also described [15].

### *7.2 Materials and Methods*

***Preparation of DNA Constructs.*** The pcDNA3-6His-SUMO1, pcDNA3-6His-SUMO2, and pcDNA3-6His-SUMO3 expression vectors were described previously [16]. The 6His-SUMO1 construct was sub-cloned into the pRSET expression vector (Invitrogen) at BamHI restriction site, while 6xHis-SUMO2 and 3 were sub-cloned at BamHI and EcoRI restriction sites. The RSFDuet-Aso1-His-Uba2 and pET23a-Ubc9 expression vectors were kind gifts from Dr. Yoshi Azuma, Department of Molecular Bioscience, University of Kansas.

***Bacterial Expression of His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 Proteins.*** Transform plasmids into *E. Coli* strain of BL21(DE3) cells, and inoculate single colony into LB medium containing ampicillin (final concentration of 200 μg/ml). Expression was induced by the addition of isopropyl-β-D-thiogalactopyranoside to a

final concentration of 1 mM when the culture reached $O.D._{600}$ ~ 0.4-0.6. The bacteria were harvested 4 hours post-induction by centrifugation at 3000 rpm for 10 minutes at 4 ℃.

***Isolation of His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 Proteins.*** The following buffers were utilized in the isolation and purification of E1, E2, SUMO1, SUMO2, and SUMO3 proteins from bacteria. Lysis buffer for E1 enzyme was prepared with 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, and 10 mM imidazole. Wash buffer for E1 enzyme was prepared with 50 mM Na-phosphate, pH 8.0, 300 mM NaCl, 20 mM imidazole, 1 mM β-mercaptoethanol, and 1 μg/ml each of aprotinin, leupeptin and pepstatin. Elution buffer for E1 enzyme was prepared with 50 mM Na-phosphate pH 8.0, 300 mM NaCl, 250 mM imidazole, 1 mM β-mercaptoethanol, and 1 μg/ml each of aprotinin, leupeptin and pepstatin. Lysis buffer for E2 enzyme was prepared with 50 mM Na-phosphate, pH 6.5, and 50 mM NaCl. Elution buffer for E2 enzyme was prepared with 50 mM Na-phosphate, pH 6.5, 300 mM NaCl, 1 mM DTT, and 1 μg/ml each of aprotinin, leupeptin and pepstatin. Lysis buffer for SUMO1, SUMO2, and SUMO3 was prepared using 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 10 mM imidazole. Wash buffer for SUMO 1, SUMO2, and SUMO3 was prepared with 50 mM Tris-HCl, pH 8.0, 100 mM NaCl, and 20 mM imidazole. Elution buffer was prepared with 50 mM Tris-HCl, pH 8.0, 50 mM NaCl, and 500 mM imidazole. Dialysis buffer was prepared with 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, and 0.05% Tween 20.

The bacterial pellet obtained from centrifugation of 5 L of bacterial culture was resuspended in 25 ml of lysis buffer and sonicated on ice for 30 s × 5. The lysates were centrifuged at 16,000 rpm for 45 minutes at 4 ℃. The supernatant was filtered with Millex-GP Filter Unit, 0.22 μm (Millipore) and stored on ice.

***Purification of His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 proteins.*** HiTrap™ Chelating HP column (GE Healthcare) containing 5 ml of resin was prepared according to the manufacturer's instructions. The resins were charged with 25 ml of 100 mM $NiSO_4$, and the column was equilibrated with 25 ml of lysis buffer. The sample was loaded onto the column, and the column was washed with 50 ml of wash buffer following sample binding. Bound proteins were eluted with 25 ml of elution buffer. The fractions with purified proteins were dialyzed against 2 L of dialysis buffer overnight at 4 ℃. Protein samples to be used for *in vitro* SUMOylation assay were concentrated with Amicon Ultra-4 Centrifugal Filter Unit, 10k (Millipore), aliquoted, and stored at -80 ℃.

***In Vitro SUMOylation Assay.*** SUMOylation assay buffer was prepared with 20 mM HEPES, pH 7.3, 110 mM potassium acetate, 2 mM magnesium acetate, 1 mM EGTA, 1 mM DTT, 0.05% Tween 20, 0.2 mg/ml ovalbumin, 1 μg/ml each of leupeptin and aprotinin. Each SUMOylation reaction contained 1 μl of RanGap1 fragment (Enzo Life Sciences Inc., Farmingdale, NY), 1 μg of E1 enzyme, 2.5 μg of E2 enzyme, 0.3 μg of Sumo1, or 1 μg of Sumo2 or Sumo3 in the presence or absence of $Mg^{2+}$-ATP. A total of 20 μl reaction volume was filled up with SUMOylation assay buffer. The

assay components were mixed and incubated at 30 ℃ for 60 minutes. The reaction was quenched by the addition of 20 μl of 2 × SDS-PAGE gel loading buffer.

***SDS-PAGE and Western Blotting.*** The presence of purified proteins was determined by 12.5% SDS-PAGE electrophoresis and Coomassie staining. To detect the sumoylated RanGap1, samples of each reaction were resolved on 10% SDS-PAGE gels and transferred to PVDF microporous membranes (Millipore), which were probed with anti-SUMO1 or anti-SUMO2/3 antibody (Enzo Life Sciences Inc.). Immunodetection was performed using the Pierce ECL Western Blotting Substrate (Thermo Scientific) according to the protocol provided by the manufacturer.

### *7.3 Results*

***Expression and Purification of His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 Proteins.*** The strategy shown in Figure 7-2 was followed to express, isolate and purify His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 proteins. The first step was to express and produce the His-tagged proteins in *E. Coli* strain of BL21(DE3) cells. Expressed proteins were harvested and lysed 4 hours after induction by isopropyl-β-D-thiogalactopyranoside. Samples were loaded onto the $Ni^{2+}$ column, which selectively retained proteins with histidine exposed on the surface of the protein. His-tagged proteins were eluted with buffer containing 500 mM imidazole and dialyzed against SUMOylation assay buffer. Purified components were collected, separated on a 12.5% SDS-PAGE gel, and visualized by staining with Coomassie blue. The His-tagged purified components had an apparent molecular mass of 38.4kD for

**Figuire 7-2**

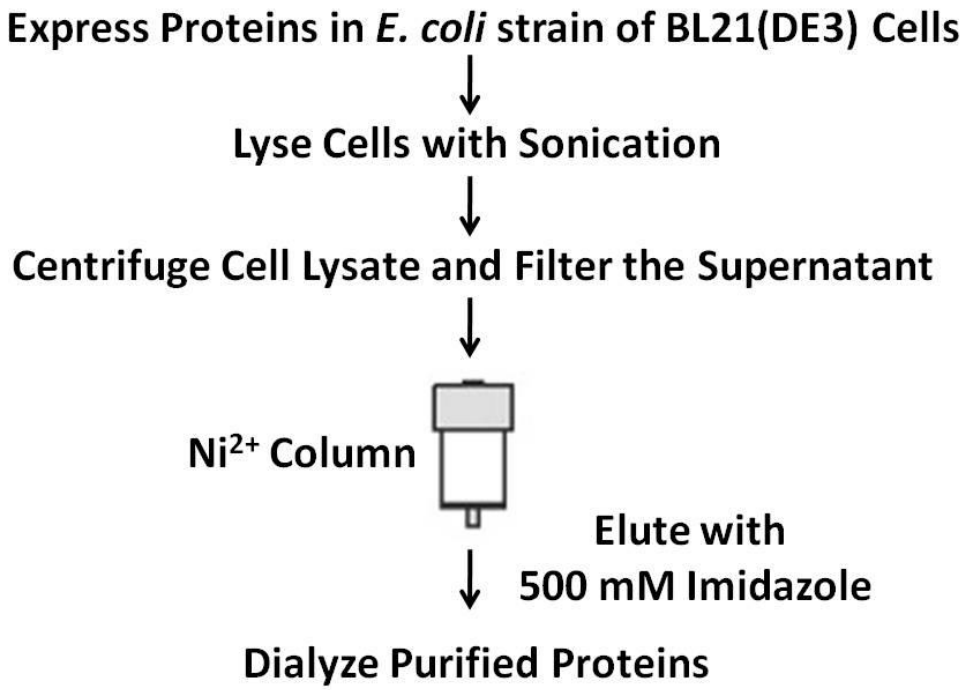


**Figure 7-2. Purification Scheme for His-tagged E1, E2, SUMO1, SUMO2, and SUMO3 Proteins.**

Aos1, 71.2kD for Uba2, and 18kD for E2 on the gel, respectively. Interestingly, the molecular weight for SUMO1 (15.1kD), SUMO2 (14.4kD), and SUMO3 (14.1kD) was shift up on SDS-PAGE (Figure 7-3).

***In Vitro SUMOylation Assays.*** RanGTPase-activating protein (RanGap1), the key regulator of Ran GTP/GDP cycle, was the first substrate identified to be post-translational conjugated with SUMO-1 in an ATP-dependent manner [17]. Here, we used RanGap1 as substrate for *in vitro* SUMOylation assays in order to exam the efficiency of the purified components. We incubated human recombinant RanGap1 fragment (418-587) together with purified E1, E2, SUMO1, SUMO2, or SUMO3 proteins in the presence and absence of the required magnesium and ATP cofactors. The extent of SUMOylation after the incubation was determined by SDS-PAGE and western blot analysis with antibodies that recognize either SUMO1 or SUMO2/3 proteins. The analysis revealed that SUMOylation of RanGap1 resulted in a ~20 kD mobility shift and RanGap1 could be modified with all three SUMO proteins in the presence of SUMO E1, E2 and $Mg^{2+}$-APT to give a single mono-sumoylated product (Figure 7-4). The presence of bands at about 35 kD can be attributed to small amounts of di-SUMO substrate, while bands at 25 kD on the western blot probed with anti-SUMO1 antibody may be due to the impurities present in the fractions containing SUMO1 component.

### *7.4 Discussion*

SUMOylation has been reconstituted *in vitro* for many known targets using

**Figure 7-3**

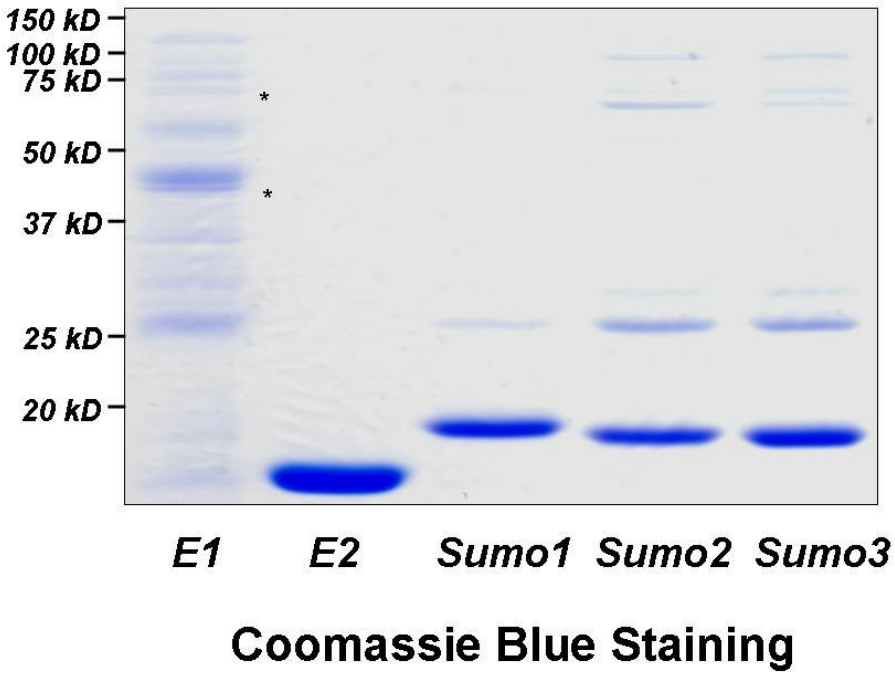


**Figure 7-3. SDS-PAGE Analysis of Purified Proteins.** 5 μg of purified component was loaded onto each lane. Proteins were separated on a 12.5% gel and visualized by staining with Coomassie blue. The two components of E1 dimer are indicated with asterisks.

**Figure 7-4**

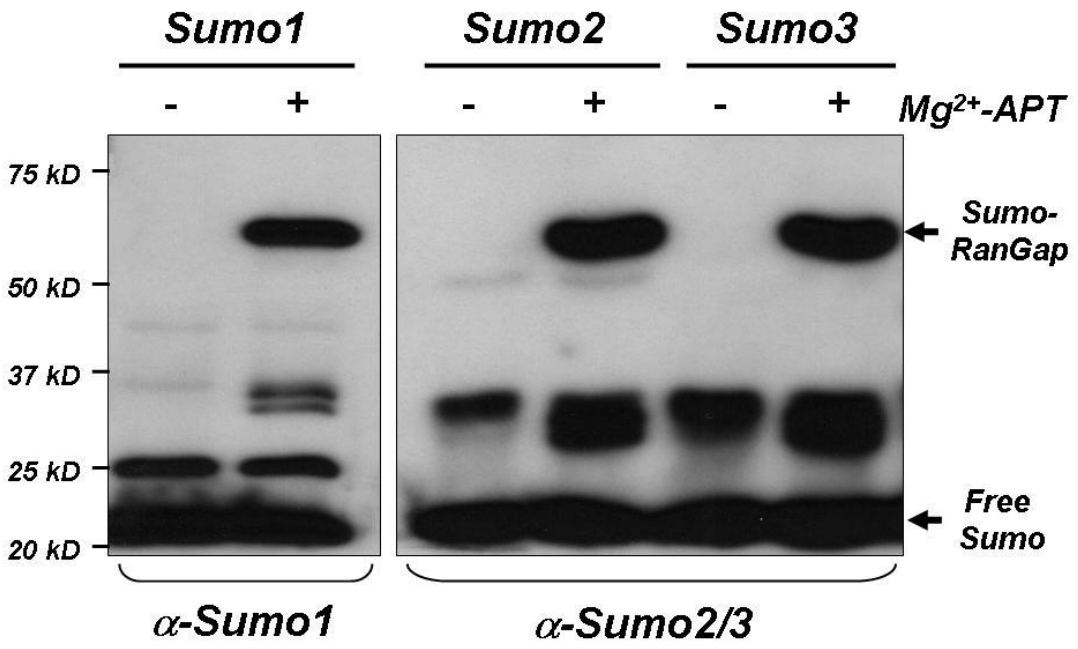


**Figure 7-4. Western Blot of *In Vitro* SUMOylation Assay for RanGap1.** 20 μl reactions containing 1 μl of RanGap1, 1 μg of E1 enzyme, 2.5 μg of E2 enzyme, 0.3 μg of SUMO1 (left panel), or 1 μg of SUMO2 or SUMO3 (right panel) were incubated at 30 ℃ in the presence or absence of $Mg^{2+}$-ATP for 1 hour. The protein was detected by western blot analysis with antibodies that recognize either SUMO1 or SUMO2/3.

recombinant SUMO enzymes and SUMO proteins purified from bacterial cells. *In vitro* SUMOylation of proteins provides a useful means for investigation of this post-translational modification. For example, it allows the identification of novel proteins as potential targets for SUMOylation pathway under *in vitro* condition, serving as a starting point for examining the role of SUMOylation *in vivo*. It can be employed to study the effect that SUMO-modification might have on specific substrate functions *in vitro*, such as protein-protein interactions, protein stability, etc. It can also be applied to identify sites, motifs, or specific amino acids of SUMO-modification within the substrates *in vitro*.

The *in vitro* SUMOylation assay we describe here does not include an SUMO E3 ligase in the reaction, which may result in a higher amount usage of Ubc9. It is the method of choice when the specific E3 ligase for a target is not known or unavailable. Even though E1 and E2 enzymes are sufficient for SUMOylation of specific substrates *in vitro*, several proteins have been demonstrated to act as SUMO E3 ligases. These enzymes allow or enhance the SUMOylation of specific target proteins under certain conditions *in vitro*. For instance, PIAS1 functions as a SUMO ligase and catalyzes the SUMOylation of p53 *in vitro* [18]. RanBP2 directly interacts with the E2 enzyme Ubc9 and strongly enhances SUMO1-transfer from Ubc9 to the SUMO1 target Sp100 [14]. Therefore, recombinant SUMO E3 ligases, such as members of PIAS family and RanBP2 can be expressed and purified from bacteria, and included in the *in vitro* SUMOylation assay. In this case, less amount of Ubc9 might be needed, but optimal Ubc9 and E3 concentrations have to be titrated for every single target protein.

$Ni^{2+}$ column, which selectively retained proteins with histidine exposed on the surface, are often used for the purification of recombinant His-tagged proteins [19]. Impurities were present in the SUMOylation components purified with $Ni^{2+}$ column, especially the E1 enzymes (Figure 7-3). Pure components can be obtained by rerunning the purified proteins from $Ni^{2+}$ column over ion exchange chromatography, namely cation exchange column (SP Sepharose) or anion exchange column (Q Sepharose). Since ion exchange column is based on adsorption and reversible binding of charged sample molecules to oppositely charged groups attached to an insoluble matrix, purification can be achieved by choosing a start buffer with a pH and ionic strength that promotes the binding of some or all contaminating substances but allows the protein of interest to pass through the column.

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# Chapter 8: The Future Outlook of PXR

## *8.1 PXR and Drug Metabolism*

Our body is constantly exposed to potentially harmful environmental xenobiotics that are ingested in the diet, inhaled, or absorbed. Although many of the water-soluable chemicals are readily eliminated from the body, lipophilic xenobiotics are particularly problematic because they often require conversion to hydrophilic molecules more suitable for excretion into urine or bile. Otherwise these lipophilic xenobiotics have the potential to accumulate to toxic concentrations over long periods of time. A complex system has been developed to defend our body against numerous xenobiotics through the combined action of the phase I oxidative cytochrome-P450 (CYP) enzymes, the phase II conjugating enzymes, and the membrane transporter proteins in liver and intestine.

The expression of genes encoding many drug-metabolizing enzymes (DMEs) and drug transporters are inducible in response to various xenobiotic compounds, representing a protective role for our body against toxic insult [1]. Not surprisingly, the importance of drug effects and toxicity through metabolism has been widely appreciated and studied. The identification and characterization of the pregnane x receptor (PXR, NR1I2) in 1998 revealed the molecular basis for this inducible defense system [2]. PXR belongs to the nuclear receptor (NR) superfamily, and is expressed predominantly in the liver and intestine. Upon activation by a variety of clinically prescribed drugs, drug metabolites, active ingredients in several widely used herbal remedies, and endobiotic compounds, PXR heterodimerizes with retinoid X receptor

alpha (RXRα, NR2B1), and up-regulates the transcription of a wide range of DMEs and transporter proteins [3]. Therefore, activation of PXR by these compounds represents the molecular basis for guarding our body against harmful assault. At the same time, however, induction of DMEs by PXR activation can also lead to accelerated metabolism of other co-administered drugs, producing potentially life-threatening drug-drug, herb-drug, and food-drug interactions in patients on combination therapy.

In the past decade, important insights have been made regarding the PXR-mediated drug-drug interactions. Up-regulation of CYP3A4 alone, the prototypical target gene of PXR, is involved in the metabolism of >50% of all prescription drugs [4]. Hence, scientists are developing *in vitro* and *in vivo* models to test drug candidates for their ability to activate PXR. The ideal drug candidates would be those that have desired therapeutic efficacy but lack PXR activity. The availability of robust, high throughput and binding assays permits the rapid identification of PXR ligands, allowing the screening of the entire library of drug candidates for PXR activity [5, 6]. Primary cultures of human hepatocytes are also employed to assess the potential for compounds to modulate the expression of PXR target genes [7]. Due to the species-specific nature of the induction of genes involved in drug metabolism and disposition, generation and characterization of humanized PXR transgenic mice exhibiting human-like response to drugs evolve as appropriate *in vivo* tools for evaluating the functions of PXR in a whole animal system [8, 9].

### *8.2 PXR and Inflammation*

In the past decade, the molecular basis for ligand-mediated PXR gene activation programs has been well established to control the metabolism and transport of xenobiotics in mammals. Interestingly, pharmacotherapy with potent PXR ligands produces several profound side effects including inflammation. It has been known for forty years that treatment with rifampicin, an antibiotics used to treat tuberculosis which is also a prototypical PXR ligand, tends to suppress humoral and cellular immunological function in liver cells in patients [10-12]. On the other hand, it has also been observed that inflammation and infection reduce hepatointestinal drug metabolism capacity [13-15]. A recent study revealed a mutual repression between steroid and xenobiotic receptors and NF-κB signaling pathways, in which PXR inhibits NF-κB-mediated reporter activity and the expression of NF-κB target genes, while activation of NF-κB reciprocally inhibits PXR activity and the expression of PXR target genes [16]. However, the specific molecular mechanism underlying these phenomena remains unknown.

Several recent reports indicate that key members of the nuclear receptor (NR) superfamily are SUMOylated to act in trans to repress the inflammatory-responses. For example, a molecular pathway has been identified in which PPAR-gamma represses the transcriptional activation of inflammatory response genes in mouse macrophages. Ligand-dependent SUMOylation of the PPAR-gamma results in targeting itself to the promoters of several inflammatory-response genes, where it inhibits gene transcription by preventing clearance of multi-protein corepressor

complexes [17]. Subsequent evidence indicates that SUMOylation is required for the suppression of STAT1-dependent inflammatory responses by LXR-alpha and LXR-beta in IFN-gamma-stimulated brain astrocytes [18]. Interestingly, the PXR protein also contains consensus SUMOylation sites, but it remains to be seen whether a similar mechanism is applicable in transrepression of NF-κB signaling by PXR protein. Recently published data from our lab has demonstrated that activation of the inflammatory response in hepatocytes strongly modulates the SUMOylation status of ligand-bound PXR. The SUMOylated PXR protein contains SUMO2/3 chains and feedback represses the immune response in hepatocytes [19]. A hypothesis has been proposed that the mechanism of selective repression of the inflammatory response is due to SUMO-modified PXR preventing clearance of multi-protein corepressor complex.

Human PXR has been implicated in the pathogenesis of inflammatory bowel diseases (IBD). PXR-mediated repression of NF-κB target genes in the colon has been shown as a critical mechanism by which PXR activation decreases the susceptibility of mice to DSS-induced IBD [20]. Rifaximin, a rifamycin analog, is a poorly absorbed oral antimicrobial agent increasingly used in the treatment of IBD. It receives new labeling for reduction in the risk of the recurrence of overt hepatic encephalopathy in patients with advanced liver disease [21, 22]. However, the mechanisms contributing to the effects of rifaximin on IBD are not fully understood. Recent studies identify rifaximin as a gut-specific human PXR ligand [23]. The preventive and therapeutic role of rifaximin on IBD is demonstrated through human

PXR-mediated inhibition of the NF-κB signaling cascade, thus suggesting that human PXR may be an effective target for the treatment of IBD [24].

### *8.3 Concluding Remarks*

Research over the past decade has made it evident that PXR is critical for regulating expression of genes that control drug metabolism and disposition. Pharmaceutical companies are now routinely screening novel compounds to determine whether they activate PXR during safety assessment studies. These screening assays should yield drugs with less potential for induction of genes linked to drug metabolism and disposition, and thereby reduce the risk for adverse drug interactions. The identification of novel ligands and target genes continues to be an important aspect of PXR research.

Recent evidence has revealed a negative regulatory role for PXR in several physiological functions including inflammation. Understanding the biochemical details and molecular mechanisms of how PXR is converted from a positive regulator of hepatic drug metabolizing enzymes into a transcriptional suppressor of inflammation in liver tissue is emerging as a key area of study for this receptor. The knowledge gained from these studies is expected to form the basis to exploit the interface between ligand-mediated PXR activation, post-translational modification of PXR, and inflammatory liver and bowel diseases, and eventually provides new opportunities for the development of novel therapeutic strategies to target this noteworthy receptor in the combat of human diseases.

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