Pregnane x Receptor is SUMOylated to Repress the Inflammatory Response

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Abstract

Long-term treatment of patients with the macrolide antibiotic and prototypical activator of pregnane x receptor rifampicin (Rif) inhibits the inflammatory-response in liver. We show here 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 be used 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.
INTRODUCTION

It has been known for forty 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 (Paunescu, 1970). This phenomenon has clinical significance, especially in HIV-infected patients presenting with co-morbid 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 recent reports indicate that key members of the NR superfamily are SUMOylated to repress the inflammatory-responses in various tissue types. Notably, Pascual et al, present a model for repression in mouse macrophages in which ligand-dependent SUMOylation of peroxisome-proliferator-activated-receptor gamma results in its recruitment to the promotors of several inflammatory-response genes where it inhibits transcription by preventing clearance of multi-protein corepressor complexes (Pascual et al., 2005). More recent 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 (Lee et al., 2009).
PXR is highly expressed in liver and is the molecular target of numerous clinically prescribed drugs, drug metabolites, active ingredients in several widely used herbal remedies (Staudinger et al., 2001; Brobst et al., 2004; Ding and Staudinger, 2005; Ding et al., 2006). Activation of hepatic PXR by these compounds represents the molecular basis of an adaptive response that both 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.

While 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 (Lichti-Kaiser et al., 2009a; Lichti-Kaiser et al., 2009b; Pondugula et al., 2009). A recent study indicates a significant increase in liver-enriched transcription factor crosstalk in patients with severe liver disease, suggesting that an elevation in the coordinate regulation of hepatic gene expression occurs during the inflammatory response (Congiu et al., 2009). Two reports have described mutually repressive and negative crosstalk between the PXR and NF-κB signaling pathways (Gu et al., 2006; Zhou et al., 2006). It is 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, though the molecular basis for these phenomena are not fully known.
Species-specific effects are often observed when examining signal transduction pathways and activating ligands of PXR (Lichti-Kaiser et al., 2009b); (Xie et al., 2000). 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-alpha (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 upon 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.
MATERIALS AND METHODS

In-vitro SUMOylation Assay.

Each SUMOylation reaction (Enzo Life Sciences) contained 1 µM recombinant purified PXR (PanVera) or RanGap1 in total 20 µl volume in the presence or absence of Mg²⁺-ATP. The assay components were mixed in a microcentrifuge tube and incubated at 30°C for 60 minutes, and the reaction was quenched by addition of 20 µl 2X-SDS-PAGE gel loading buffer. To detect the SUMOylated proteins, 5 µl sample of each reaction was resolved using 10% SDS-PAGE, and the immunoblot analysis was conducted using anti-SUMO1 or anti-SUMO2/3 antibodies (Enzo Life Sciences). The membrane was stripped and re-probed using monoclonal anti-PXR H-11 monoclonal antibody (Santa Cruz Biotechnology).

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, UK). Plasmids p3258 (pCMV-hUBC9) and p3259 (pCMV-hUBC9 C93S) were obtained from Addgene. HeLa cells were maintained in DMEM media 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 (Golebiowski et al., 2009). For transfection assays, HeLa cells were grown in 6-well dishes for 24 h until 80% confluence. Cells were transfected with the expression plasmids using Lipofectamine 2000 (Invitrogen). Forty-eight hr post-transfection, cells were washed twice with PBS and harvested in 200 µl lysis buffer (6 M Guanidinium-HCl, 10 mM Tris, 100 mM sodium
phosphate buffer pH 8.0). Following sonication the cell lysates were cleared by centrifugation at 3,000 g for 15 min. The cleared cell lysates were mixed with 25 µl Ni²⁺-linked agarose (Qiagen) that had been pre-washed 3X in cell lysis buffer. The mixture was incubated for 2 h on a rotator at room temperature and centrifuged for 2 min at 1,000 rpm to gather the beads. The beads were washed once in wash buffer I (8 M urea, 10 mM Tris, 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, 50 mM sodium phosphate buffer pH 6.75). The beads were re-suspended in 40 µl 2X-SDS-PAGE gel loading buffer, boiled for 5 min and 20 µl samples were resolved using 10% SDS-PAGE. The gel was transferred to PVDF 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 (Brobst et al., 2004). In brief, Hela cells were plated in 96-well plates at a density of 7000 cells per well. Cells were transfected using Lipofectamine 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. To measure NF-κB activation, cells were transfected with CMV-β-gal (20 ng), NF-κB-Luc reporter gene (20 ng), pSG5-hPXR (10 ng), 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 using CV-1 cells. Briefly, cells were transiently
transfected with CMV-β-gal (20 ng), XREM-Luc (20 ng), pSG5-hPXR (5 ng), and pBluescript was added to achieve 110 ng of total DNA per well. Twenty-four hours post-transfection cells were treated with either vehicle (0.1% DMSO) or drug (10 μM Rif) for an additional 24 hr. Treatment of cells with TNFα was accomplished using 10ng/ml. Luciferase activities were determined using a standard luciferase assay system (Promega, Madison, WI). Beta-galactosidase activities were determined by the 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 (Brobst et al., 2004; Lichti-Kaiser and Staudinger, 2008). Hepatocytes were plated in collagen-coated 6-well plates at a density of 8 × 10^5 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 PCN, 10 ng/ml TNFα, or 25 μM MG132 for 24 h.

**Immunoprecipitation of Human PXR Protein.**

Following 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 1X protease inhibitors (Thermo Scientific). Cell lysates were pre-cleared with 20 μl of immobilized protein A (Repligen). Immunoprecipitation of the human PXR protein was accomplished 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. Following SDS-PAGE, the protein was transferred to PVDF membrane (Millipore Bioscience Research Reagents) that was probed with a monoclonal anti-PXR antibody and a rabbit monoclonal anti-SUMO2/3 antibody (Cell Signaling). Immunodetection was performed using the Pierce ECL western blotting substrate or SuperSignal west femto maximum sensitivity substrate (Thermo Scientific) according to the protocol provided by the manufacturer.

**RNA Isolation and Real Time Quantitative-PCR Analysis.**

Total RNA was isolated from mouse liver or cell culture using a commercially available reagent, TRIzol (Invitrogen), according to the manufacturer's directions. After DNase I treatment, 1 ug of RNA was reverse-transcribed using random primers following the manufacturer's instruction (Promega, Madison, WI). Equal amounts of cDNA were used in real-time quantitative polymerase chain reaction (rt-QPCR) reactions. Reactions included 1 × SYBR Green (Lonza Rockland, Inc., Rockland, ME) and 300 nM primers specific for each gene. The primer sets were designed using the Primer3 program (http://frodo.wi.mit.edu/). The sequences (5' to 3') for the primers are as follows: 18S; forward primer 5'-AGTCCCTGCCCTTTGTACACA-3', reverse primer 5'-CGATCCGAGGGCCTCATA-3'; Cyp3a11, forward primer 5'-CAAGGAGATGTTCCCTGTCA-3', reverse primer 5'-CCACGTTCACTCCAAATGAT-3'; IL-1β, forward primer 5'-TTCCAGGATGAGGACATGAG-3', reverse primer 5'-TTCTGTCCATTGAGGTGGAG-3'. Cycling conditions were 95°C for 2 min followed by 45 cycles of 95°C for 15 s, 60°C for 15 s, and 68°C for 15 s using the Cepheid Smart
Cycler system (Sunnyvale, CA). The fold induction was calculated as described previously (Staudinger et al., 2003).
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 (Pikarsky et al., 2004; Greten et al., 2005). We previously developed a genetically engineered line of mice that lack the Pxr gene (PXR-KO) (Staudinger et al., 2001). 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 real-time quantitative polymerase chain reaction (rt-QPCR) showed that the expression levels of these inflammatory cytokines were significantly increased in the livers of PXR-KO mice (Figure 1). In particular, the IL-1β gene expression level was dramatically increased (~20-fold) in the livers isolated from the PXR-KO mice when compared to 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 crosstalk between the PXR and NF-κB in liver.

We next examined IL-1β gene expression levels following treatment of primary cultures of hepatocytes isolated from either wild type or PXR-KO mice with pregnenalone 16α-carbonitrile (PCN), TNFα, or both PCN and TNFα. Hepatocytes were treated with the prototypical rodent PXR activator PCN for 48 hr, and then with TNFα for an additional 12 hr. Treatment of wild type hepatocytes with PCN alone produced significant repression of IL-1β mRNA expression (Figure 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 was elevated in PXR-KO hepatocytes (Figure 2B). TNFα treatment produced a significant increase in the level of IL-1β mRNA that was effectively repressed by co-treatment 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 when compared to wild type hepatocytes (Figure 2A and B, 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 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 following the incubation was determined using SDS-PAGE and western blot analysis with commercially available antibodies that recognize either SUMO1 or SUMO2/3.
proteins (Figure 3B, left panel) or with antibodies that recognize the human PXR protein (Figure 3B, right panel). 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. Recent studies confirm that the Ubc9 enzyme can effectively catalyze the formation of poly-SUMO chains in vitro (Capili and Lima, 2007; Knipscheer et al., 2007). The functional significance of the formation of poly-SUMO chains on PXR is currently unknown. While 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 over-expression 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 utilized cDNA expression vectors encoding 6x-His-tagged SUMO1, SUMO2, and SUMO3 proteins together with an expression vector that encodes the human PXR protein. Co-transfection of HeLa cells with the PXR expression vector together with the 6x-His-SUMO1, 6x-His-SUMO2, or 6x-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 would likely cleave SUMOylated forms of PXR upon cell lysis are rapidly de-activated under these denaturing conditions.
Using this experimental approach we detected SUMOylated PXR using a western blot with the α-PXR monoclonal antibody (Figure 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 4A, lanes 6 and 7). Importantly, addition of an expression vector encoding the dominant-negative Ubc9 (C93S) dramatically reduced poly-SUMO chain formation (Figure 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 4B, left panel, lanes 3-5). Importantly, the same blot was stripped and re-probed with the α-SUMO2/3 antibody and produced confirmatory results that reveal a decrease in SUMO3 immunoreactivity (Figure 4B, right panel, lanes 3-5). These data demonstrate specific conjugation of poly-SUMO3 chains to the human PXR protein in cultured cells. Since forced over-expression 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.
The Endogenous PXR Protein is SUMOylated in Response to TNFα in Cultured Hepatocytes.

While 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 (Dennis and O'Malley, 2005)]. 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 immuno-precipitation of cell extracts using a well characterized custom anti-human PXR polyclonal antibody (Lichti-Kaiser et al., 2009b). 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. Importantly, this experimental approach also enabled us to perform important control experiments using cross-detection with both anti-PXR as well as 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 5A, middle panel). When the blot was stripped and re-probed with the anti-SUMO2/3 antibodies, we detected a band of the identical size that was enriched following treatment with MG132 (Figure 5A, bottom panel), 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 have created a novel line of ‘humanized’ PXR transgenic mice that express the FLAG-tagged version of the protein exclusively in liver (Lichti-Kaiser and Staudinger, 2008). Primary cultures of both wild type and transgenic ‘humanized’ PXR hepatocytes were treated with vehicle, rifampicin, TNFα, or TNFα plus rifampicin for 24 hr. Immunoprecipitation with the anti-hPXR polyclonal antibody followed by western blot with a monoclonal anti-PXR antibody was performed. The 70 kD band was increased by treatment with rifampicin, TNFα, and co-treatment with rifampicin and TNFα exclusively in the transgenic ‘humanized’ PXR mice (Figure 5B). It is already established that the PXR-KO mice have elevated levels of TNFα and related inflammatory cytokines (Zhou et al., 2006). Thus, treatment of ‘humanized’ PXR mice, which lack expression of murine PXR in small intestine, with rifampicin alone increased SOMOylation in this model, likely due to 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 5A and 5B 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 hr. Total cell extract was subjected to standard pre-clearing methods and subsequent immunoprecipitation techniques using the anti-hPXR polyclonal antibody. As before, equal loading was determined using an aliquot of whole cell lysate and western blotting to detect β-actin (Figure 5C, top panel). Subsequent western blot analysis with a monoclonal α-SUMO2/3 rabbit antibody detected a band at the expected size of SUMOylated PXR protein (~70 kD) (Figure 5C, bottom panel). Treatment of cells with TNFα alone or with 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 (-TGGGGACCTTCCGC-) multimerized five times. Previous publications from our laboratory utilizing transient transfection and PXR in 96-well reporter gene assays were performed using CV-1 cells (Ding and Staudinger, 2005; Lichti-Kaiser et al., 2009a). Importantly, treatment of cultured CV-1 cells with TNFα produced an approximate 10-fold increase in NF-κB reporter gene activity, while treatment with Rif did not have any effect upon NF-κB reporter gene alone or in combination with 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 TNF\(\alpha\)-mediated NF-\(\kappa\)B reporter gene activity (Figure 6A).

Titration of the dominant-negative Ubc9 (C93S) expression vector (5, 10, and 25 ng per
well) restored TNF\(\alpha\)-mediated increases in NF-\(\kappa\)B reporter gene activity in a dose-
dependent manner (Figure 6B). Interestingly, expression of SUMO3 and Ubc9 had no
effect upon PXR-mediated gene activation when directed towards the PXR-response
element-controlled luciferase reporter gene (Figure 6C).
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 AP-1 transcription factor (Jonat et al., 1990; Helmberg et al., 1995). 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 (Calleja et al., 1998). However, additional studies were unable to corroborate this finding (Ray et al., 1998). Thus, the molecular basis of Rif-mediated suppression of inflammation has remained obscure. While two other recent publications have identified the existence of strong repressive crosstalk between the PXR- and NF-κB-signaling pathways (Gu et al., 2006; Zhou et al., 2006), no well defined molecular mechanism for Rif-mediated repression of the inflammatory response in hepatocytes was identified. The data presented here are consistent with a recent 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 (Fiorucci et al.). 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 appear 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. While SUMOylation controls a large number of cellular processes, it clearly plays a prominent role in repression of transcription (Gill, 2005). 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 (Iniguez-Lluhi and Pearce, 2000). 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 (Holmstrom et al., 2003; Shiio and Eisenman, 2003). Interestingly, in this context the SUMO-2/3 proteins exhibited greater repression when compared with SUMO-1 (Holmstrom et al., 2003). While SUMO-1 appears to be mostly conjugated to proteins, the SUMO-2/3 proteins are primarily found in a free form. However, an increase in SUMO-2/3 incorporation into substrates is
detected following exposure to several stress conditions including heat shock (Golebiowski et al., 2009). 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 PPAR-mediated repression, a molecular mechanism for this repression could include selective targeting of PXR to NR corepressor NCoR/HDAC3 complexes on inflammatory gene promoters (Pascual et al., 2005). A working model for this hypothesis is shown in Figure 7.

PXR regulates key aspects of drug metabolism and drug transporter activity in several key tissue types including liver, intestine, and in capillary endothelial cells that comprise the blood-brain barrier (Staudinger et al., 2001; Staudinger et al., 2003; Ott et al., 2009; Xu et al., 2009). PXR is the molecular target of numerous clinically prescribed drugs, drug metabolites, active ingredients in several widely used herbal remedies, and endobiotic compounds (Staudinger et al., 2001; Brobst et al., 2004; Ding and Staudinger, 2005; Lichti-Kaiser and Staudinger, 2008). Activation of PXR by these compounds represents the molecular basis of an adaptive response that both 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 knockout (PXR-KO) mouse model. A study by Teng et al. revealed that PXR-KO mice exhibit significant diminution of endotoxin-mediated
suppression of the expression of the \textit{Mrp2} gene in liver (Teng and Piquette-Miller, 2005). More recently, investigations have revealed that the activation of NF-kappaB (NF-\(\kappa\)B) and PXR somehow produces transrepression of the expression of each others target genes, respectively (Zhou et al., 2006). Interestingly, this study showed that the PXR-KO mice exhibit elevated markers of inflammation in the small bowel when compared with wild type mice, including significantly increased expression levels of several key NF-\(\kappa\)B-target genes including COX-2, IL-6, TNF\(\alpha\), IL-2, IL-1\(\alpha\), IL-1\(\beta\), IL-15, and ICAM-1. Disruption of the molecular interaction between PXR and DNA through increased protein-protein interaction between the p65 subunit of NF-\(\kappa\)B and retinoid x receptor has been proposed as the molecular basis for transrepression of the xenobiotic response by inflammatory cytokines (Gu et al., 2006), though the precise mechanism that gives rise to the selective interaction between these two proteins is not currently known. Several recent studies indicate that PXR-mediated inhibition of NF-\(\kappa\)B is required for anti-fibrogenic effects and repression of CYP3A4 expression in hepatocytes (Yang et al.; Axon et al., 2008). 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.
REFERENCES


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Legends for Figures

Figure 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 using rt-QPCR. Data are expressed as relative expression in PXR-KO mice as compared with that observed in wild type mice and are normalized to 18S. * = p <0.01.

Figure 2. Co-treatment 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 hr and then treated with 10 μM PCN for 48 hr before the addition of TNFα (10 ng/ml) for an additional 12 hr. Total RNA was collected and the relative expression level of IL-1β mRNA was determined using rt-QPCR. Data are expressed as relative expression (Log10 scale) as 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 3. (A) 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, as well as three others that are predicted as ‘low probability’ SUMOylation sites. (B) In Vitro SUMOylation of Human PXR Protein. The human PXR protein was used as a test substrate for SUMO1, SUMO2, and SUMO3. The protein was detected using western blot analysis with antibodies that recognize either SUMO1 or SUMO2/3 (left panel). The RanGap protein was used as a positive control for experimental integrity. The same blot was stripped and re-probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein (right panel).

Figure 4. (A) Detection of SUMOylated Human PXR Protein in HeLa Cells. The human PXR protein was co-expressed 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 using nickel-linked agarose beads. The blot was probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein. (B) Dominant-negative Ubc9 (C93S) Protein Inhibits SUMOylation of Human PXR in a Dose-dependent Manner. The human PXR protein was co-expressed in HeLa cells together with 6x-His-tagged SUMO3 and increasing amounts of dominant-negative Ubc9 (C93S). SUMOylated protein was purified using nickel-linked agarose beads. The blot was probed for PXR immunoreactivity using a monoclonal antibody that recognizes the human PXR protein (Left Panel). The blot was stripped and re-probed with an antibody that recognizes SUMO2/3 (Right Panel).
Figure 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 hr 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 Panels). 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 (H-11 monoclonal, SantaCruz) or SUMO2/3. The asterisk (*) indicates cross-reaction with the secondary antibody due to the presence of heavy chain.

Figure 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), Ubc9 (10 ng/well). Luciferase activity was determined using standard methods and is reported as fold-induction ± S.E.M. and was normalized to β-galactosidase activity. (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, respectively). Luciferase activity was determined using standard methods and is reported as fold-induction ± S.E.M. and was normalized to β-galactosidase. (C) CV-
Figure 7. **Model of PXR-mediated Repression of Inflammatory and Xenobiotic Response Pathways.** The TNFα-mediated inflammatory response strongly modulates SUMOylation of ligand-bound PXR protein to actively repress the expression of inflammatory response genes.
**Figure 1**

Relative mRNA Expression

- **Wild Type**
- **PXR-KO**

- TNFα
- IL-6
- IL-1α
- IL-1β

* indicates statistical significance.
Figure 3A

Consensus SUMOylation Sequence

-Ψ-K-x-D/E-

Ψ - hydrophobic residue
x - any amino acid

...101 KCLESG MKKE MIMSDEAVEE RRALIK RKKKS ERTGTQPLGV QGLTEEQRM
151 IRELMDAQMK TFDTTFSHFK NFRPGVLSSG ...

Site 1 Site 2 Site 3 Site 4

DBD hinge Ligand-binding

- High Probability - Low Probability

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Figure 3B

**Sumo1**  **Sumo2**  **Sumo3**

| Mg²⁺-ATP | + | - | + | - | + | - |

![Image showing protein bands for Sumo1, Sumo2, Sumo3 with corresponding protein markers (RanGap, PXR), and antibody labels (α-Sumo1, α-Sumo2/3, α-PXR).](image-url)
Figure 4A

His-Sumo1 + PXR
His-Sumo3 + PXR
His-Sumo2 + PXR

In Vitro

No DNA
PXR
Ubc9
Ubc9 (C93S)
Ubc9
Ubc9 (C93S)
Sumo1
Sumo2
Sumo3

Poly-Sumo-PXR
Sumo-PXR
PXR

Nickel Beads \(\rightarrow\) WB: \(\alpha\)-PXR
**Figure 4B**

**His-Sumo3 + PXR**

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**Nickel Beads → Western Blot**

**α-PXR**

**α-Sumo2/3**
Figure 5A

**Input**

- **Veh**
- **MG132**

**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 5B

**Wild Type**

- Veh
- Rif
- TNFα

**PXR\textsubscript{tg}**

- Veh
- Rif
- TNFα

**IP:** α-hPXR (polyclonal)

**WB:** α-hPXR (monoclonal)

**Input**

- 150 kD
- 100
- 75
- 50

**Sumo-hPXR**

**FLAG-hPXR**
Figure 6A

NF-κB Response Element

Luc

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Fold Induction
Figure 6B

**NF-κB Response Element**

**Luc**

**Fold Induction**

- Veh
- Rif
- Veh
- Rif
- Veh
- Rif
- Veh
- Rif
- Veh
- Rif
- Veh
- Rif

**DOMINANT NEGATIVE Ubc9 (C93S)**
Figure 6C

PXR Response Element

Luc

Fold Induction

veh | Rif | veh | Rif | veh | Rif | veh | rif | veh | Rif | veh | rif

- | TNFα | - | TNFα | - | TNFα | - | TNFα

PXR | PXR + SUMO3 | PXR + SUMO3 + UBC9
Figure 7

Hepatocyte

TNFα

+ Rifampicin

nuclear membrane

Sumo3

PXR

NF-κB

Su3

Su3

Active Repression

Inflammatory-Response Genes