Pregnane X Receptor Is SUMOylated to Repress the Inflammatory Response

Gang Hu, Chenshu Xu, and Jeff L. Staudinger

Department of Pharmacology and Toxicology, University of Kansas, Lawrence, Kansas

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ABSTRACT

Long-term treatment of patients with the macrolide antibiotic and prototypical activator of pregnane X receptor (PXR) 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 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 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 (Păunescu, 1970). 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., (2005) 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 (Lee et al., 2009).

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 (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 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 (Lichti-Kaiser et al., 2009a,b;

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ABBREVIATIONS: NR, nuclear receptor; PXR, pregnane X receptor; hPXR, humanized PXR; RIF, rifampicin; TNF α , tumor necrosis factor α ; PAGE, polyacrylamide gel electrophoresis; RT-QPCR, real-time quantitative polymerase chain reaction; PCN, pregnenolone 16 α -carbonitrile; KO, knockout; IL, interleukin; CMV, cytomegalovirus; NF- κ B, nuclear factor κ B; LUC, luciferase; MG132, *N*-(benzyloxycarbonyl)leucinyllleucinyllleucinal Z-Leu-Leu-Leu-al.

Pondugula et al., 2009). 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 (Congiu et al., 2009). Two reports have described mutually repressive and negative cross-talk between the PXR and NF- κ B signaling pathways (Gu et al., 2006; Zhou et al., 2006). 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 (Xie et al., 2000; Lichti-Kaiser et al., 2009b). 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 (TNF α) 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- κ B 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- κ B target genes and the hepatic inflammatory response during therapy with Rif.

Materials and Methods

In Vitro SUMOylation Assay. Each SUMOylation reaction (Enzo Life Sciences Inc., Farmingdale, NY) contained 1 μ M recombinant purified PXR (PanVera Corp., Madison, WI) or RanGap1 in total 20- μ l 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 min, and the reaction was quenched by the addition of 20 μ l of 2 $\times$ SDS-PAGE gel loading buffer. To detect the SUMOylated proteins, a 5- μ l 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 (Golebiowski et al., 2009). 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 μ l 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 μ l of Ni²⁺-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 μ l of 2 $\times$ SDS-PAGE gel loading buffer and boiled for 5 min, and 20- μ l 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- κ B-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 by using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. To measure NF- κ B activation, cells were transfected with CMV- β -galactosidase (20 ng), NF- κ B-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 μ M Rif) for an additional 24 h. Treatment of cells with TNF α was accomplished by using 10 ng/ml TNF α . 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 (Brobst et al., 2004; Lichti-Kaiser and Staudinger, 2008). Hepatocytes were plated in collagen-coated six-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 μ M Rif, 10 μ M pregnenalone 16 α -carbonitrile (PCN), 10 ng/ml TNF α , or 25 μ M *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 $\times$ protease inhibitors (Thermo Fisher Scientific, Waltham, MA). Cell lysates were precleared with 20 μ l 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 μ g 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 $\times$ 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'-AGTCCCT-GCCCTTTGTACACA-3', reverse primer 5'-CGATCCGAGGGCCT-CACTA-3'; Cyp3a11, forward primer 5'-CAAGGAGATGTTCCCT-GTCA-3', reverse primer 5'-CCACGTTCACTCCAAATGAT-3'; and IL-1 β , 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 (Sunnyvale, CA) Smart Cycler system. 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- κ B 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- κ B target genes. Analysis of the expression levels of genes encoding TNF α , IL-6, IL-1 α , and IL-1 β using RT-QPCR showed that the expression levels of these inflammatory cytokines were significantly increased in the livers of PXR-KO mice (Fig. 1). In particular, the IL-1 β gene expression level was dramatically increased ($\sim$ 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 pro-

Fig. 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 TNF α , IL-6, IL-1 α , and IL-1 β 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. *, $p < 0.01$.

vide supporting evidence for establishing the existence of transcription factor cross-talk between the PXR and NF- κ B in liver.

We next examined IL-1 β gene expression levels after treatment of primary cultures of hepatocytes isolated from either wild-type or PXR-KO mice with PCN, TNF α , or both PCN and TNF α . Hepatocytes were treated with the prototypical rodent PXR activator PCN for 48 h, and then with TNF α for an additional 12 h. Treatment of wild-type hepatocytes with PCN alone produced significant repression of IL-1 β mRNA expression (Fig. 2A). In contrast, treatment of PXR-KO hepatocytes with PCN did not repress expression of IL-1 β . As expected, the expression level of IL-1 β was elevated in PXR-KO hepatocytes (Fig. 2B). TNF α treatment produced a significant increase in the level of IL-1 β mRNA that was effectively repressed by cotreatment of wild-type hepatocytes with TNF α and PCN. PCN-mediated repression of TNF α -inducible IL-1 β expression was completely absent from hepatocytes isolated from PXR-KO mice. Moreover, the fold in-

Fig. 2. Cotreatment of wild-type hepatocytes with PCN and TNF α represses expression of IL-1 β 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 μ M PCN for 48 h before the addition of TNF α (10 ng/ml) for an additional 12 h. Total RNA was collected, and the relative expression level of IL-1 β 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 ($p < 0.01$).

crease of IL-1 β mRNA expression produced by TNF α was dramatically elevated in PXR-KO hepatocytes compared with wild-type hepatocytes (Fig. 2, note the scales). These data indicate an active and suppressive role for liganded PXR in regulating the expression of IL-1 β mRNA in response to TNF α .

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 (Fig. 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 (Fig. 3B, left) or with antibodies that recognize the human PXR protein (Fig. 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 (Capili and Lima, 2007; Knipscheer et al., 2007). 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 $\times$ 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 $\times$ His-SUMO1, 6 $\times$ His-SUMO2, or 6 $\times$ 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 (Fig. 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 ex-

Fig. 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 and 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. 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.

Fig. 4. A, 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. B, 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.

amine SUMOylation of endogenous PXR in primary cultures of hepatocytes.

The Endogenous PXR Protein Is SUMOylated in Response to TNF α 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 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 immunoprecipitation 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. 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) (Fig. 5A, 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 (Fig. 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 (hPXR_{tg}) 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 (Lichti-Kaiser and Staudinger, 2008). Primary cultures of both wild-type and transgenic humanized PXR hepatocytes were treated with vehicle, rifampicin, TNF α , or TNF α 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 TNF α and cotreatment with rifampicin and TNF α exclusively in the transgenic humanized PXR mice (Fig. 5B). It has already been established that the PXR-KO mice have elevated levels of TNF α 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, probably because of the presence of increased levels of inflammatory cytokines such as TNF α or IL-1 β . 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 TNF α treatment when expressed in mouse hepatocytes. Taken together, the data presented in Fig. 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- κ B transcriptional activity in human hepatocytes. Primary cultures of human hepatocytes were therefore treated with vehicle, Rif, TNF α , or both Rif and TNF α 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 (Fig. 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) (Fig. 5C, bottom). Treatment of cells with TNF α alone or TNF α together with Rif produced an increased level of

Fig. 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 μ M), TNF α (10 ng/ml), or TNF α + Rif. Whole-cell protein lysates were subjected to SDS-PAGE and blotted with antibodies against β -actin to ensure equal loading of the

SUMOylated PXR. These results indicate that TNF α produces increased levels of detectable SUMOylated PXR protein in human hepatocytes.

SUMOylation of PXR Represses TNF α -Inducible NF- κ B Reporter Gene Activity. Our experiments using cultures of hepatocytes provide compelling evidence that TNF α 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- κ B-luciferase reporter gene strategy. This reporter gene contains an NF- κ B-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 (Ding and Staudinger, 2005; Lichti-Kaiser et al., 2009a). It is noteworthy that treatment of cultured CV-1 cells with TNF α produced an approximate 10-fold increase in NF- κ B reporter gene activity, whereas treatment with Rif did not have any effect on NF- κ B reporter gene alone or in combination with TNF α . 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 α -mediated NF- κ B reporter gene activity (Fig. 6A). Titration of the dominant-negative Ubc9 (C93S) expression vector (5, 10, and 25 ng per well) restored TNF α -mediated increases in NF- κ B reporter gene activity in a dose-dependent manner (Fig. 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 (Fig. 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 activator protein-1 transcription factor (Jonat et al., 1990; Helmlberg 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. Although two other articles have identified the existence of strong repressive cross-talk between the PXR and NF- κ B 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 review article that highlights the increased recognition of the counter-regulatory role of several liver- and intestine-en-

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.

Fig. 6. Functional significance of SUMO3 modification of the PXR protein. A, CV-1 cells were transfected with an NF- κ B-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 $\pm$ 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). Luciferase activity was determined by using standard methods, is reported as fold-induction $\pm$ S.E.M., and was normalized to β -galactosidase. C, CV-1 cells were transfected as in A except a PXR-response element luciferase reporter gene (XREM-LUC) was used. Veh, vehicle.

riched NR proteins in entero-hepatic immune responses (Fiorucci et al., 2010). 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 (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 (Iñiguez-Lluhí 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; Shio and Eisenman, 2003). It is noteworthy that in this context the SUMO-2/3 proteins exhibited greater repression compared with SUMO-1 (Holmstrom et al., 2003). 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 (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- κ B 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 (Pascual et al., 2005). A working model for this hypothesis is shown in Fig. 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 (Staudinger et al., 2001, 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

Fig. 7. Model of PXR-mediated repression of inflammatory and xenobiotic response pathways. The $\text{TNF}\alpha$ -mediated inflammatory response strongly modulates SUMOylation of ligand-bound PXR protein to actively repress the expression of inflammatory response genes.

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 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 (2005) 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- κ B and PXR somehow produces transrepression of the expression of each other's target genes (Zhou et al., 2006). 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- κ B target genes, including cyclooxygenase 2, IL-6, $\text{TNF}\alpha$, IL-2, IL-1 α , IL-1 β , 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- κ 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), 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- κ B is required for antifibrogenic effects and repression of CYP3A4 expression in hepatocytes (Axon et al., 2008; Yang et al., 2010). 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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Address correspondence to: Jeff L. Staudinger, Pharmacology and Toxicology, University of Kansas, 1251 Wescoe Hall Dr., 5038a Malott Hall, Lawrence, KA 66045. E-mail: stauding@ku.edu