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Acetylation of Pregnane X Receptor protein determines selective function independent of ligand activation

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Abstract

Pregnane X Receptor (PXR), like other members of its class of nuclear receptors, undergoes post-translational modification [PTM] (e.g., phosphorylation). However, it is unknown if acetylation (a major and common form of protein PTM) is observed on PXR and, if it is, whether it is of functional consequence. PXR has recently emerged as an important regulatory protein with multiple ligand-dependent functions. In the present work we show that PXR is indeed acetylated *in vivo*. SIRT1 (Sirtuin 1), a NAD-dependent class III histone deacetylase and a member of the sirtuin family of proteins, partially mediates deacetylation of PXR. Most importantly, the acetylation status of PXR regulates its selective function independent of ligand activation.

Keywords

Pregnane X Receptor; orphan nuclear receptor; acetylation-deacetylation; SIRT1 deacetylase; ligand-independent function

Introduction

Post-translational modifications (PTM) of many eukaryotic proteins often regulate and extend their range of functions. Lysine acetylation has emerged as a major PTM for many histone and also non-histone proteins, thus playing a crucial role in various nuclear as well as cytosolic processes [1]. Within the acetylproteome, the pattern and effects of lysine acetylation have been found to be diverse, context-dependent and to differ from protein to protein. It has, moreover, been suggested that in response to diverse cellular signaling, lysine acetylation can cross-talk with other PTMs thus forming dynamic regulatory programs [1]. Lysine acetylation was first reported for histone proteins and the modifying enzymes involved were named histone acetyltransferases (HAT) and histone deacetylases (HDAC) accordingly as they regulated acetylation or deacetylation respectively [1]. But recent findings suggest that their substrates are not limited to histones. Many transcription factors, including nuclear receptors (NRs) like AR, LXR, FXR, ER α have been reported to be regulated by acetylation [2;3;4]. SIRT1, a mammalian ortholog of the yeast Sir2 protein, is a

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Disclosure Statement

None

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class III HDAC that has been reported to deacetylate many target proteins, (including a few NRs), either activating or repressing their functions in the process [3;4]. Of the various NRs known, the biology of the orphan nuclear receptor, Pregnane X receptor (PXR) has evolved to be much more complex and subtle than initially understood. PXR or NR1I2, belongs to the nuclear hormone receptor superfamily of transcription factors containing ligand- and DNA-binding domains [5]. Initially described as a xenobiotic sensor critical for the transcriptional regulation of genes central to detoxification pathways, PXR has now emerged as a regulatory protein with multiplicity of roles (including cellular detoxification, inflammation and cancer) and is being revealed as behaving in a tissue-specific manner [6]. Till date, all PXR functions have been attributed to ligand activation. PXR is known to possess the broadest ligand specificity of the NR superfamily, by virtue of its large, spherical, and flexible ligand-binding pocket and, thus, a structurally diverse array of compounds is able to activate PXR [6]. But ligand activation alone cannot be the sole determinant of PXR activation states since it has already been shown that phosphomimetic PXR mutants are transcriptionally repressed compared to basal wild-type PXR. In this context as an example, signaling mediated by growth factor insulin represses PXR-mediated CYP promoter activity through induction of the PI3K-protein kinase B (PKB or Akt) pathway, and the forkhead in rhabdomyosarcoma (FKHR or FOXO1) transcription factor [7;8]. This implies that non-ligand dependent signals may play a significant role in activation or repression of these receptors. There seems to exist an additional hitherto-unexplored complex regulatory program that mediates context-dependent PXR activation. In this paper we sought to find out whether additional PTMs like acetylation may govern PXR activation/repression. Here we show that PXR is acetylated and that the SIRT1 protein is presumably responsible for partial deacetylation of PXR, and, also, that acetylation status of PXR regulates its selective function (i.e. lipogenesis) independent of ligand activation.

Materials and Methods

Cell lines

The human colon cancer cell line LS174T, human liver carcinoma cell line HepG2 and human 293T cells [American Type Culture Collection (ATCC)] and cultured according to their recommendations.

Reagents and Antibodies

Cell culture media, charcoal adsorbed fetal bovine serum (FBS), dimethyl sulfoxide (DMSO), rifampicin (rif), pregnenolone carbonitrile (PCN), 9-cis-retinoic acid (RA), nicotinamide (NAM), resveratrol (res), trichostatin A (TSA) and Nile Red were purchased from Sigma Aldrich (St. Louis, MO). Effectene transfection reagent and Ni-NTA-agarose were purchased from Qiagen (Valencia, CA). PXR H-160 antibody (sc-25381) used for immunoprecipitation and PXR N-16 antibody (sc-9690) used for western blot analysis were from Santa Cruz Biotechnology (Santa Cruz, CA). A rabbit polyclonal anti-PXR antibody (against full length PXR) was kindly gifted by Prof. R. K. Tyagi (Jawaharlal Nehru University, India). Acetylated-lysine antibodies (#9441 and #9681) were from Cell Signaling Technology (Danvers, MA). SIRT1 antibody was purchased from Millipore. Bovine anti-goat antibody, goat anti-mouse and anti-rabbit antibodies and non-specific rabbit IgG were also purchased from Santa Cruz Biotechnology.

Plasmid constructs

The pcDNA3.1-His-tagged human PXR (hPXR) expression construct was kindly provided by Dr. Petr Pavlek (Charles University, Prague). It was generated by PCR amplification of cDNA encoding amino acids 1-434 of human PXR (kindly provided by Dr. S. Kliewer of UT Southwestern Medical Center to Dr. Petr Pavlek) into pcDNA3.1/His(C) vector

(Invitrogen). Flag-SIRT1 construct (Addgene plasmid 1791) was purchased from Addgene Inc. (Cambridge, MA) [9]. Expression constructs for human PXR variants 1, 2 and 3 were kind gifts from the laboratory of Dr. Peter Mackenzie (Flinders University, Australia) [10]. The constitutively active PXR mutant constructs, namely, S247W single mutant or S247W, S208W, C284W triple mutant (TM) have been described before [11].

Cell culture and Transfection

293T, LS174T and HepG2 cells were cultured according to ATCC recommendations. For expression of different plasmid constructs, 293T cells were seeded 18–24 h before transfection and grown to 75–80% confluence. Transfections were carried out using Effectene transfection reagent following the manufacturer's protocol. The transfection efficiency varied from 50–70% of the total cell population.

Preparation of Nuclear and Cytoplasmic fractions from mammalian cell extracts

Cells were harvested at 4° C and re-suspended in buffer NE (10 mM HEPES pH 7.9, 100 mM KCl, 0.5 mM EDTA, 1 mM DTT, 1 mM PMSF, 1.5 mM MgCl₂), containing a cocktail of protease inhibitors. The suspension was incubated on ice for 15 min, lysed by adding 1% NP-40 followed by vortexing for 10 sec. The lysate was immediately centrifuged at 1000 g for 5 min and the postnuclear supernatant ("Cytosolic Fraction") was kept at 0–4° C. The nuclear pellet was re-suspended in the same volume of NE buffer as that of the cytosolic fraction and the re-suspended nuclei were vortexed, briefly sonicated, incubated on ice for 30 min and centrifuged at 1000 g for 5 min. The clarified supernatant was designated as the "Nuclear Fraction".

Affinity purification of His-tagged PXR from Ni-NTA column

Small scale purification of His-tagged PXR from nuclear extracts of 293T cells was carried out following a procedure as previously described [12]. Briefly, nuclear extracts were incubated with equilibrated Ni-NTA resin for at least 2 h at 4°C to allow binding of His-tagged proteins to the resin. The beads were then extensively washed with wash buffer containing 50–80 mM imidazole and finally the expressed His-PXR was eluted with 300 mM imidazole.

Western blot analysis

Western blot analysis was carried out following the usual procedure. The Western bands were scanned and analyzed by densitometry using Image J software (National Institutes of Health, Bethesda, MD). Final results were plotted as bar graphs in Microsoft Excel or GraphPad Prism 5. In each case, data are the average of three individual experiments. The error bars show the standard deviation from the average value.

Immunoprecipitation of PXR from nuclear extracts

Nuclear extracts were immunoprecipitated using rabbit anti-PXR antibody (H-160) or normal rabbit IgG (negative control). Immunoprecipitation samples were immunoblotted with goat anti-PXR antibody (N-16) and mouse anti-acetyl lysine antibody.

Nile Red assay in primary hepatocytes

Primary hepatocytes were isolated from PXR wild type and knock-out mice (C57BL/6 background provided by Dr. Wen Xie) following a procedure as described before [11]. The cells were treated as indicated and then fixed in 3% paraformaldehyde. Nile Red staining was carried out following a procedure as described before [13]. Cells were examined under fluorescence microscope and Nile Red staining was expressed as an increase in total cellular

fluorescence (pixel number per average fluorescence intensity) per cell. Statistical analysis was done using ANOVA test.

Lenti-based shRNA knock down of PXR

This was carried out following a procedure as described in [14]. The knock down efficiency was about 80%.

Results

PXR is acetylated in vivo

To investigate whether PXR is acetylated, we transfected 293T cells either with a pcDNA-His-hPXR expression construct or with only the pcDNA-His vector plasmid (control) and subjected lysates to western blots using goat polyclonal anti-PXR antibody (PXR N-16), followed by rabbit polyclonal anti-acetyl lysine antibody. PXR expressed through transfection was found to be acetylated under normal culture conditions (Fig. 1A). We next determined whether endogenous PXR is acetylated. For this purpose, endogenous PXR was immunoprecipitated from nuclear fraction of HepG2 cells (using rabbit polyclonal anti-PXR antibody H-160), and acetylated PXR was detected by Western blotting using goat polyclonal anti-PXR antibody N-16 and mouse polyclonal anti-acetyl lysine antibody (Fig. 1B). Endogenous PXR was also found to be acetylated in lysates from LS174T cells (Fig. 1C). The acetylated band corresponding to PXR disappeared when PXR was knocked down with PXR specific shRNA, but not with scrambled shRNA (Fig. 1C). Additionally, 293T cells transfected with different splice variants of human PXR (namely, PXR1, PXR2 and PXR3), showed that all the variants are acetylated (data not shown).

Activation of PXR by PXR/RXR ligands stimulate deacetylation

293T cells, transfected with pcDNA-His-hPXR expression construct, were incubated with either DMSO (vehicle), or with 20 μ M of hPXR ligand rifampicin (rif), or with 20 μ M rif and 1 μ M of RXR ligand 9-cis retinoic acid (RA) for 48 h and the cells were fractionated into cytosolic and nuclear extracts. Nuclear extract from each set was passed through Ni-NTA column, His-PXR was eluted (as described in Materials and Methods), and subjected to Western blot analysis using goat polyclonal anti-PXR antibody (PXR N-16), followed by rabbit polyclonal anti-acetyl lysine antibody. It was found that activation of PXR by rifampicin alone, significantly stimulated deacetylation (and/or inhibited acetylation). Activation by PXR/RXR ligands (rif, as well as RA) promoted further deacetylation of the protein, as illustrated in Fig. 2, panels A and B. Next, 293T cells were transfected with either wild type PXR or constitutively active PXR mutant constructs, namely, S247W single mutant or S247W, S208W, C284W triple mutant (TM), and corresponding nuclear extracts were subjected to Western blot analysis, using rabbit polyclonal anti-PXR antibody (obtained from Tyagi lab) and rabbit polyclonal anti-acetyl lysine antibody. It was found that relative acetylation levels were significantly lower in the constitutively active PXR mutant species compared to the wild type (Fig. 2, panels C and D). Similarly, when HepG2 cells were incubated for 48 h with PXR/RXR ligands (20 μ M rif and 1 μ M RA) and endogenous PXR was immunoprecipitated, its acetylation level was found to be significantly lower than PXR immunoprecipitated from DMSO-treated cells (Fig. 2, panels E and F).

SIRT1 partially regulates PXR deacetylation

To investigate whether SIRT1 regulates PXR deacetylation, we transfected 293T cells with FLAG-tagged SIRT1 expression construct and/or pcDNA-His-hPXR construct and subjected the cells to 48 h of treatment with PXR/RXR ligands. Affinity purification of His-

PXR from nuclear extracts of each cell population and subsequent Western blot analysis, using both anti-PXR and anti-acetyl lysine antibodies, showed that while overexpression of SIRT1 had a modest (but reproducible) effect on PXR deacetylation, the effect was much more pronounced in the presence of PXR/RXR ligands (Fig. 3, panels A and B). This prompted us to determine whether SIRT1 associates with PXR. As shown in Fig. 3C, transfected SIRT1 protein from 293T cells co-purified with His-PXR in a Ni-column eluate only when the two proteins were co-expressed, indicating that SIRT1 could interact with PXR, directly or indirectly. Interestingly, SIRT1 was present in the Ni column eluate of His-PXR both in the presence and absence of PXR/RXR ligands, suggesting that the interaction might be ligand independent. To analyze the specificity of SIRT1 on deacetylation of PXR, we decided to treat HepG2 cells with the SIRT1 inhibitor nicotinamide (NAM), [15], or SIRT1 activator resveratrol (res), [16], and look at the acetylation status of endogenous PXR. For this, we first checked if NAM or res acts as ligands for PXR. We found that NAM (and also TSA, trichostatin A, inhibitor of other HDACs) does not act as PXR ligand (on PXR transactivation assays) while res acts as a very weak ligand at high concentrations (data not shown). However, res was found to be cytotoxic for the cells if incubated for more than 6 h (data not shown). So, accordingly, HepG2 cells were incubated with either DMSO (vehicle) or 20 μ M rif (control) or 10mM NAM or 40 μ M res for 6 h and fractionated into cytosolic and nuclear extracts. Endogenous PXR from nuclear extract of each set was immunoprecipitated and subjected to western blot analysis to study its acetylation status. It was found that while treatment with resveratrol for 6 h led to significant deacetylation of PXR, treatment with NAM for the same time period led to a modest but reproducible increase in its acetylation status (suggesting a modest inhibition of PXR deacetylation) (Fig. 3, panels D and E). This indicated that acetylation level of PXR is sensitive to activation and inactivation of SIRT1 protein by res and NAM respectively. To find out if a longer incubation with NAM can produce a more marked effect on PXR deacetylation, we incubated 293T cells transfected with His-PXR construct with 10mM NAM and/or 0.5 μ M TSA for 16 h. The expressed His-tagged hPXR was purified from the nuclear extract by affinity purification using Ni-NTA column and was subjected to western blot analysis using both anti-PXR and anti-acetyl lysine antibodies. It was found that a longer incubation indeed augmented the effect of NAM on PXR deacetylation (Fig. 3, panel F, lane 2), but TSA had a stronger effect on it (Fig. 3, panel F, lane 3), while NAM and TSA together could inhibit PXR deacetylation even further (Fig. 3, panel F, lane 4). This suggested that SIRT1 is responsible for partial deacetylation of PXR, but clearly, other HDAC(s) are also involved in it as evident from our experiment with TSA described above (Fig. 3, panels F and G). When endogenous PXR was immunoprecipitated from LS174T cells treated for 6 h with res, or NAM or NAM+TSA, SIRT1 was similarly found to be partially regulating PXR acetylation status (Fig.3, panels H and I).

Acetylation regulates selective function of PXR independent of ligand activation

As discussed previously, it is feasible that PXR has a role independent of ligand activation. More specifically, we wanted to see if PXR acetylation, by itself, can regulate selective functions of the protein. PXR has been reported to have an impact on lipid homeostasis in liver [17]. Lipogenesis or lipid accumulation can be very conveniently followed by using the fluorescent dye, Nile Red which specifically stains lipid droplets [13]. Thus, such a system was quite suitable for our study. Primary hepatocytes were isolated from wild type (WT) and PXR knock-out (KO) mice and incubated with either the mouse PXR ligand PCN or with res, or NAM and/or TSA. It was found that PCN- treated wild type hepatocytes showed much more intense Nile Red staining compared to the wild type hepatocytes treated with DMSO, suggesting increased lipogenesis or lipid accumulation in response to PXR ligand. At the same time, treatment with res only, also showed similar increased Nile Red staining and hence, lipogenesis, compared to DMSO-treated cells. Treatment with NAM and/or TSA

resulted in lower Nile Red staining in the wild type hepatocytes (Fig. 4, panels A and B). On the other hand, Nile Red staining in hepatocytes from PXR knock-out mice remained insensitive to PCN, res, NAM and TSA, as the staining pattern remained the same under the various conditions (treatments) used. This indicated the phenotype to be PXR specific. (Fig. 4, panels C and D). Thus, the Nile Red study in hepatocytes suggested that PXR acetylation status, alone, can regulate the lipogenic pathway, independent of any ligand. We also used hepatocytes from mice expressing constitutively active PXR for similar Nile Red staining under the various conditions described above. In all these hepatocytes, the Nile Red staining pattern was found not only to be similar but also to be high in all the experimental sets, including the DMSO treated cell population (data not shown).

Discussion

This is the first report of adopted orphan nuclear receptor, PXR, being post-translationally modified by acetylation. Indeed this phenomenon is not unique to PXR but has been demonstrated for other adopted orphan nuclear receptors, LXR, FXR, VDR, PPAR and its coregulators (SRC-1, PGC-1) [2;3;4]. PXR activation results in deacetylation of the protein. We have shown, as in the case of both LXR and FXR, SIRT1 is associated (directly or indirectly) with PXR and is partially responsible for deacetylation. However, other deacetylases (e.g., HDACs) are also likely to impact on PXR during activation. Finally, perhaps the most provocative finding is that partial deacetylation per se (using resveratrol) seems sufficient to induce PXR mediated lipogenic phenotype in primary hepatocytes *in vitro*. In this context, it is important to note that resveratrol is not a significant PXR agonist (data not shown).

While it is clear that PXR is basally acetylated and that it deacetylates upon activation, it remains unclear as to which lysine residues are important for acetylation. Indeed, deriving mutants that ablate acetylation or enhance the “acetylator” phenotype is then a crucial next step in determining and validating acetylation as it pertains to PXR. Also, the complexes (e.g., specific HATs) involved in PXR acetylation is unknown and PXR deacetylation can only be partially attributed to SIRT1 activation. At the molecular level the exact mechanism by which SIRT1 induces this effect remains unknown. Indeed, SIRT1 could either perform this function directly on PXR or its coregulators may mediate SIRT1 action or both. Other deacetylases may play an important role in enhancing or assisting with deacetylation upon PXR activation (e.g., HDACs). Future studies will focus on defining the molecular basis for these interactions and further quantify the absolute effect that acetylation/deacetylation has on PXR phenotypes.

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The abbreviations used are

PXR	Pregnane X Receptor
PTM	post-translational modification
SIRT1	sirtuin1
NR	nuclear receptor

HDAC	histone deacetylase
HAT	histone acetyltransferase
DMSO	dimethyl sulfoxide
Rif	rifampicin
PCN	pregnenolone carbonitrile
RA	9-cis-retinoic acid
NAM	nicotinamide
Res	resveratrol
TSA	trichostatin A

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Fig. 1.

PXR is acetylated *in vivo*. (A) Nuclear extracts from 293T cells, transfected either with a pcDNA-His-hPXR expression construct or pcDNA-His vector plasmid (control), were affinity purified from a Ni-NTA column and subjected to Western blot analysis. (B) PXR was immunoprecipitated from nuclear fraction of HepG2 cells and subjected to Western blot analysis. (C) LS174T cell lysates expressing either scrambled shRNA or PXR shRNA were subjected to Western blot analysis. The same blot was also probed for β -actin (loading control).

Fig. 2.

PXR activation stimulates deacetylation. (A) 293T cells, transfected with pcDNA-His-hPXR expression construct, were incubated with either DMSO, or with 20 μ M of hPXR ligand rifampicin (rif), or with 20 μ M rif and 1 μ M of RXR ligand 9-cis retinoic acid (RA), as indicated, and the cells were fractionated into cytosolic and nuclear extracts. Nuclear extract from each set was passed through Ni-NTA column to elute His-tagged PXR and subjected to Western blot analysis. (B) PXR acetylation level in the transfected 293T cells after each indicated treatment, as presented in panel A, was quantified with respect to eluted PXR protein level in each set and finally normalized to DMSO-treated set. (C) 293T cells were transfected with either wild type (WT) PXR or constitutively active PXR mutant constructs, namely, S247W single mutant or S247W, S208W, C284W triple mutant (TM), and corresponding nuclear extracts were subjected to Western blot analysis. The same blot was also probed for β -actin (loading control). (D) Acetylation level of expressed wild type and mutant PXR proteins, as presented in panel C, was quantified with respect to PXR protein level in each set and finally normalized to the wild type set. (E) PXR was immunoprecipitated from HepG2 cells, incubated with DMSO or PXR/RXR ligands, as

indicated, and subjected to Western blot analysis. (F) Acetylation level of PXR protein immunoprecipitated from HepG2 cells after each indicated treatment, as presented in panel E, was quantified with respect to amount of immunoprecipitated PXR protein in each set and finally normalized to DMSO-treated set. Each experiment was performed three separate times. Histogram, *mean*; error bars $\pm$ *SD*.

Fig. 3.

Deacetylation of PXR is mediated partially by NAD-dependent SIRT1 deacetylase. (A) 293T cells, transfected with FLAG-tagged SIRT1 expression construct and/or pcDNA-His-hPXR construct, were treated for 48 h with either DMSO or with PXR/RXR ligands, as indicated. Following Ni-column affinity purification of His-PXR, each eluate was subjected to western blot analysis. (B) PXR acetylation level in the transfected 293T cells after each indicated treatment, as presented in panel A, was quantified with respect to eluted PXR protein level in each set and finally normalized to DMSO-treated PXR-transfected set. (C) 293T cells were transfected with combinations of FLAG-tagged SIRT1 expression construct and pcDNA-His-hPXR construct. The cells were treated for 48 h with either DMSO or with PXR/RXR ligands, as indicated. This was followed by Ni-column affinity purification of His-tagged PXR from nuclear extracts of each cell population and subsequent Western blot analysis. (D) PXR was immunoprecipitated from nuclear extracts of HepG2 cells treated for 6 h with DMSO, or rif, or res, or NAM (as indicated), and subjected to western blot analysis. (E) Acetylation level of PXR protein after each indicated treatment, as presented in panel D, was quantified with respect to amount of immunoprecipitated PXR protein in each set and finally normalized to DMSO-treated set. (F) 293T cells transfected with His-PXR construct were treated with DMSO, or NAM and/or TSA for 16 h. The expressed His-tagged hPXR was purified from the nuclear extract by affinity purification using Ni-NTA column and was subjected to western blot analysis. (G) PXR acetylation level in the transfected 293T cells after each indicated treatment, as presented in panel F, was quantified with respect to eluted PXR protein level in each set and finally normalized to DMSO-treated set. (H) Endogenous PXR was immunoprecipitated from nuclear extracts of LS174T cells treated for 6 h with DMSO/NAM/res/NAM+TSA (as indicated), and subjected to western blot analysis using anti-PXR and anti-acetyl lysine antibodies. (I) Acetylation level of PXR protein after each indicated treatment, as presented in panel H, was quantified with respect to amount of immunoprecipitated PXR protein in each set and finally normalized to DMSO-treated set. Each experiment was performed three separate times. Histogram, *mean*; error bars $\pm$ *SD*.

Fig. 4.

PXR acetylation status regulates lipogenesis independent of ligands. (A) Primary hepatocytes were isolated from wild type (WT) mice and incubated with either the mouse PXR ligand PCN or with res, or NAM and/or TSA, as indicated. Nile red staining was carried out with these cells and (B) data quantified (* $p < 0.000.1$, ** $p < 0.000.1$, 1 way ANOVA). Bar, 10 μm . (C) Primary hepatocytes were isolated from PXR knock-out (KO) mice and incubated with either the mouse PXR ligand PCN or with res, or NAM and/or TSA, as indicated. Nile red staining was carried out with these cells and (D) data quantified (* $p = 0.62$, 1 way ANOVA). Bar, 10 μm . Each experiment was performed three separate times. Histogram, *mean*; error bars $\pm$ *SD*.