An evaluation of the anti-inflammatory properties of the pregnane X receptor gene isoforms PXR1 and PXR3

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Abstract
The pregnane X receptor (PXR) is a nuclear receptor (NR) that primarily activates genes involved in drug metabolism. However, PXR also suppresses inflammation. This study investigated the anti-inflammatory activity of PXR1 and the minor isoform PXR3. Luciferase reporter experiments showed that like wtPXR1, PXR1 mutants and wtPXR3 that are transcriptionally inactive suppressed pro-inflammatory gene expression. This suggests that PXR uses distinct regions and by extension mechanisms to induce and repress gene induction. This study hypothesised that PXR represses inflammation via a mechanism called transrepression. One crucial feature of transrepression is conjugation with Small Ubiquitin-like Modifier (SUMO) proteins. Pull-down assays showed that both wtPXR1 and wtPXR3 are targets for conjugation with SUMO1, -2 and -3 proteins. The mutagenesis of putative SUMO conjugation sites revealed that residues K170 and K108 within PXR1 and PXR3, respectively, are important for their transrepressive activity. Collectively, these findings provide further insight into the anti-inflammatory properties of PXR.

Keywords
inflammation, mutagenesis, PXR, PXR3, SUMOylation, transrepression

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Background
The pregnane X receptor (PXR) is a nuclear receptor (NR) that is expressed mainly in the liver and intestines and functions to regulate drug metabolism. The NR1I2 gene comprises nine exons and produces three main PXR isoforms: PXR1, PXR2 and PXR3.1,2 PXR1 is the major isoform. Unlike the other isoforms, its function and mechanisms of action are well characterised. Structurally, the ligand binding domain (LBD) of PXR1 is distinct from that of other NRs. The LBD is markedly spacious, flexible and adopts an archetypal α-helical triple layered sandwich comprising 10 α-helices. Surrounding this, are five β-sheets (two are unique to PXR). Also, residues 175–235 add extra flexibility to the LBD.

PXR2 is very similar to PXR1 in function and activates many genes involved in drug metabolism. Structurally, however, it possesses an extra N-terminal 39 residues as compared to PXR1.1,2 Alternative splicing produces PXR3 that is missing residues 174–210 of the full length PXR1 protein and therefore lacks many of the unique features of the PXR1-LBD1,2 (Figure 1(a)). PXR3 is transcriptionally inactive and has no known ligands or function.

Apart from its role in drug metabolism, PXR has anti-inflammatory capabilities.1,3,4 PXR and the pro-inflammatory mediator NF-κB share an antagonistic relationship and serve to regulate each
For example, the p65 unit of NF-κB has been shown to prevent PXR1 activation, which inhibits induction of PXR target genes. Conversely, PXR-null mice exhibit significantly increased expression of NF-κB target genes, pointing to its importance in regulating NF-κB activity. Little is known about how PXR switches between the different functions of transactivation and repression (drug metabolism and inflammation respectively). However, PXR routinely represses gene induction
at atypical promoters using a mechanism called transrepression that involves PXR associating with other regulatory proteins and transcription factors. Conjugation of NRs with Small Ubiquitin-like Modifier (SUMO) is typical for many transrepression mechanisms. SUMOylation is a post-translational modification that is similar to ubiquitin. SUMO is also covalently conjugated to lysine residues on target proteins. Unlike ubiquitin however, SUMOylation is believed to act as a switch that changes NR function.

The first aim of this study was to investigate the mechanisms used by PXR1 to perform its respective transactivation and repressive functions. Transcriptionally inactive PXR1 ‘transactivation’ mutants were created: R98M (cannot bind DNA), T57A/T57D (impaired nuclear localisation) and Y2257 (impairs PXR homodimerization therefore activation). The ability of the PXR1 transactivation mutants and later wt PXR3 to activate and repress gene induction was compared to that of wtPXR1 using luciferase assays. Although incapable of activating gene induction, both the PXR1 ‘transactivation’ mutants and wtPXR3 repressed pro-inflammatory gene induction.

The second aim was to investigate the relationship between SUMO and PXR-mediated repression. Pull-down assays were used to examine SUMO conjugation with PXR1 and also PXR3, given its aforementioned repressive ability. In addition, putative SUMO conjugation sites within PXR1/PXR3 were mutated. The repressive activity of these mutants was compared with that of wtPXR1. This study gave further insight into the anti-inflammatory activity of PXR.

Materials and methods

Isolation of hepatic primary cells, RNA isolation and reverse transcription

Hepatocytes for molecular cloning were obtained from healthy human liver sections after resection surgeries. Hepatocytes were isolated using a modified two-step collagenase perfusion method as described previously. Human liver tissue was obtained with full ethical approval from University of Nottingham Hospitals National Health Service (NHS) Trust Biobank (ethical approval number: 04/Q2403/70; NHS R+D approval no: (Q) GS070403). Collagenase was from Serva (NB 4G grade). RNA was isolated using the Tri Reagent method (T9424, SIGMA, USA), then purified using the NucleoSpin® RNA Clean-up kit (Macherey Nagel) according to the manufacturers’ specifications. RNA was reverse transcribed using AffinityScript Reverse Transcriptase (Agilent Technologies). PXR1/PXR3 cDNA was amplified using Phusion High Fidelity Polymerase (M0530S, New England Biolabs, USA). The cycling parameters were 98°C, 30 s (1 cycle), 98°C, 30 s followed by 69°C, 1 min (35 cycles), 69°C, 30 min (1 cycle) and 4°C hold, using the Gene Amp PCR System 9700 Thermocycler.

Directional TOPO cloning of PXR1 / PXR3 and molecular cloning of cDNA/promoter constructs

The sequences of the primers used were RXRα: FWD: 5′-ggatccatggacaccaaatctctg, REV: 5′-gg atcccaatggacaccaaatctctg-3′; cJUN: FWD: 5′gcataacatgctacaagatggc-3′; PBREM: FWD: 5′-cg ggtgccgtgcaatggccacc-3′; REV: 5′-cgctgacgtcgcttcagttgggagcc-3′; XREM: FWD: 5′-ctttctccattatagtccagatggc-3′; REV: 5′-ctttctccattatagtccagatggc-3′; AP1: FWD: 5′-gggtatgcagacaacgactgctgctg-3′; REV: 5′-gggtatgcagacaacgactgctgctg-3′. The pENTR/D-TOPO kit (K2400-20, Invitrogen, USA) was used to clone PXR1, PXR3 and PIASγ cDNA into the pcDNA3.1/nV5-DEST vector according to kit instructions. This created N-terminal V5-tagged constructs. RXRα and cJUN cDNA were cloned into pCMVTAG1 mammalian expression vector and pHA-UBC9 was a kind gift from Gao et al., Case Western Reserve University, USA. Using sequential subcloning events into the pGL4.10[LUC2] vector (Promega), the PXREM reporter which is activated by both Rifampicin (RIF) and Phenobarbital was created. It contained the 158bp MinTK Minimum Thymidine Kinase promoter (MinTK), the XREM (Xenobiotic Enhancer Responsive Module) and the PBREM (Phenobarbital Responsive Enhancer Module). The XREM and PBREM were amplified from the promoter region of the CYP2B6 gene (regions −29292 to −28903 and −3428 to −3188, respectively).

The pro-inflammatory promoters described below were used to investigate PXR-mediated repression. The interleukin (IL)-8 reporter was a gift from Dr Thilo Hagen via Dr Simon Dawson (University of Nottingham) and contained NF-kB response elements targeted by p52 homodimers. The iNOS reporter (Dr Simon Dawson University...
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of Nottingham) contains kB elements responsive to p50:p65 NF-kB heterodimers and is highly inducible by lipopolysaccharide (LPS). The AP-1 reporter10 contains a unique 21 nucleotide sequence from the Human α2(I) Collagen (COL1A2) gene (region −5098 to −5062) which was cloned into the pGL4.10[luc2] vector and is specifically responsive to cfos:junB heterodimers.

Site directed mutagenesis

The putative PXR1 and PXR3 mutants (transactivation and SUMO) were created using the respective pcDNA3.1/nV5-DEST constructs as templates. The QuickChange® Lightning Site-Directed Mutagenesis Kit (210519, Agilent, USA) was used as instructed by the manufacturer. The putative PXR1 SUMOylation sites K70, K108 and K129 were identified using the SUMO site prediction software SUMOsp 2.0. The previously identified K170 site was also chosen.11 These lysines on the surface of the PXR1/3 proteins were mutated to arginine (R).

Transfection and luciferase reporter assay

Xtremegene HP transfection reagent (6366244001, Merck, USA) was used as directed by the manufacturer (Roche). DNA was transfected into RAW 264.7 cells using a 1:1 ratio of Xtremegene:DNA. The reporter constructs (PXREM, IL-8, iNOS and AP1; 250 ng each) were co-transfected with 125 ng each of PXR1/PXR3 (and mutants), RXRα and cJUN. Where indicated, cells were treated with 10 µM RIF (R3501, SIGMA, USA) and 1 µg/mL LPS (L2630, SIGMA, USA), for 18 h at 37°C, then harvested using the Luciferase Assay System (E1500, Promega, USA) as instructed.

His tag pull-down assay and western blotting

The pull-down assay was performed as previously described12 using HeLa cells. Cells were co-transfected with wtPXR1/PXR3 or SUMO mutants, His-tagged SUMO1, −2, −3 (a gift from Dr Simon Dawson) and HA-UBC9, and V5-PIASγ where indicated. An aliquot of the lysate (input sample) was removed to determine protein concentration using the Pierce™ BCA Protein Assay Kit. The Ni²⁺-agarose system (30210, Qiagen, Germany) was used to isolate SUMOylated PXR1/PXR3 proteins according to kit instructions. About 20 µg of input samples (I) and 45 µL of the final pull-down sample (P) were used for immunoblotting using mouse anti-hPXR antibody (sc-48340, Santa Cruz Biotechnology, USA) and the reference protein rabbit anti-Cyclophilin-B antibody (ab16045, Abcam, UK), both at 1:1000 concentrations. The secondary antibodies used were the IR CW 800 Goat anti-mouse and the IR CW 680 Goat anti-rabbit (Li-COR, USA), both at 1:10000 concentrations. PXR and cyclophilin-B proteins were detected using the Odyssey infrared imaging system (LI-COR), allowing for simultaneous detection of both PXR and cyclophilin proteins on the same blot.

Data analysis

Statistical analysis was performed with GraphPad Prism 8.01 software, using one-way analysis of variance (ANOVA) followed by Bonferroni’s post hoc test (n = 3 represents three independent experiments performed in triplicate). All data represent mean values ± SEM unless otherwise stated.

Results

Transcriptionally inactive PXR proteins repressed pro-inflammatory gene induction

The relationship between the transactivation and repressive functions of PXR1 was investigated. Luciferase assay results showed that unlike wtPXR1, the transcriptionally inactive PXR1 transactivation mutants could not induce PXREM activity in the presence of RIF (Figure 1(b)). However, they significantly and comparably repressed induction of the LPS-induced IL-8 promoter, as compared with wtPXR1 (Figure 1(c)). Surprisingly, both wtPXR3 and its transactivation mutants repressed LPS-induced IL-8 activity (Figure 1(e)). Furthermore, both wtPXR1 and wtPXR3 significantly repressed gene induction at the distinct iNOS and AP-1 pro-inflammatory promoters (Figure 1(f) and (g)). It was noted that PXR1-mediated repression was more significant than that of PXR3-mediated
investigating the involvement of SUMO in the repressive activity of PXR

SUMOylation is an important component of the transrepression mechanism employed by NRs. Pull-down assays using cell lysate from transfected HeLa cells revealed that both PXR1 and PXR3 were substrates for all three SUMO proteins (SUMO1, SUMO2 and SUMO3). For PXR1 and PXR3 blots, respectively, PXR proteins were not detected in the absence (−) of HIS-tagged SUMO proteins (lanes 7, 11 and 15 on each blot). The molecular weight (MW) of PXR is approximately 50KDa, but higher MW bands (>50KDa) in the lanes with SUMO2 and SUMO3 were also observed. This may indicate poly-SUMO2/3 chains and/or simultaneous SUMOylation events occurring at different sites. This was not observed in the presence of SUMO1 because it cannot form poly-SUMO chains. Blots were probed with an anti-PXR antibody to detect PXR1 and PXR3 proteins and an anti-cyclophilin-B as a control. I = Input samples, P = pull-down conditions. Luciferase assays revealed that (c) unlike the other SUMO mutants, only K170R was significantly less capable of repressing LPS-induced IL-8 expression than wtPXR1. (d) Likewise, compared with wtPXR3, only K108R was significantly less able to repress LPS-induced IL-8 reporter expression. Luciferase assay conditions were normalised to and presented as a percentage of the mean of the respective control (IL-8 + LPS). Statistical analysis was performed using one-way ANOVA followed by Bonferroni’s post hoc test (****p < 0.001, n = 3 represents three independent experiments done in triplicate; +++P < 0.001 vs PXR1-LPS; ####p < 0.001 vs PXR3-LPS).

Discussion

This study investigated the mechanism used by PXR proteins to suppress pro-inflammatory gene induction. The first experiments examined the relationship between the activation and repression roles of PXR1. Residues R98, Y225 and T57 are essential for PXR1-mediated transactivation owing to their involvement in DNA binding, PXR1 and K108R mutants were significantly less repressive of LPS-induced IL-8 induction as compared with wtPXR1 and wtPXR3, respectively (Figure 2(c) and (d)). These sites, therefore, may be important to the repressive role of these PXR isoforms.
homodimerization and nuclear localisation, respectively.\textsuperscript{1,5,6,7,11} Despite being transcriptionally inactive, the PXR1 transactivation mutants and wtPXR3 significantly repressed IL-8 promoter activity. This suggests that the processes of PXR1 homodimerization, coactivator recruitment and DNA binding, while critical to PXR’s transactivation function, are not as vital to its repression function. It is hypothesised, therefore, that drug metabolism and anti-inflammatory activities involve distinct PXR1 residues/domains, therefore mechanisms. Studies into the anti-inflammatory activity of PXR focus mainly on PXR1. Given the lack of research into the role of other isoforms, these results suggest a possible role of PXR3 in the repression of inflammation.

In addition, PXR3-mediated repression was less significant as compared with that of wtPXR1 suggesting that residues 174–210 which are missing in PXR3\textsuperscript{1,2} are important to the repressive capability of PXR1.

SUMOylation is an important process in many NR-related transrepression mechanisms.\textsuperscript{4} The conjugation of PXR1 with all three SUMO proteins supports previous observations.\textsuperscript{3} However, the SUMOylation of PXR3 is an interesting new find. In addition, K170 and K108 were shown to be important to the repressive function of PXR1 and PXR3, respectively. Their influence on this repressive role may be owing to their location in the protein. K170 and K108 are located in the LBD and hinge region, respectively. Besides its role in ligand binding, the LBD influences conformational changes that determine whether a NR becomes an activator or repressor of gene induction.\textsuperscript{1} Also, like the LBD, the hinge region is important for the recruitment and association with co-repressors.\textsuperscript{1} Moreover, acetylation is another post-translational modification which acts as a precursor to SUMOylation thereby facilitating PXR-mediated repression.\textsuperscript{4} Like SUMO, acetylation targets lysines in proteins. Therefore, the identified SUMO sites may also serve as acetylation sites.

In conclusion, these findings provide further scope for investigating how PXR proteins switch from gene induction to gene repression. If this process is understood, then the innate anti-inflammatory properties of PXR can be harnessed to develop new strategies and drugs to suppress pro-inflammatory signalling pathways.

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