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Pregnenolone 16α-carbonitrile ameliorates concanavalin A-induced liver injury in mice independent of the nuclear receptor PXR activation

Susumu Kodama[a,b,*], Takuto Shimura[a], Hideaki Kuribayashi[a], Taiki Abe[a], Kouichi Yoshinari[a,c]

[a] Division of Drug Metabolism and Molecular Toxicology, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-Aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan
[b] Laboratory of Pharmacotherapy of Life-Style Diseases, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-Aoba, Aoba-ku, Sendai, Miyagi 980-8578, Japan
[c] Department of Molecular Toxicology, School of Pharmaceutical Sciences, University of Shizuoka, 52-1 Yada, Suruga-ku, Shizuoka, Shizuoka 422-8526, Japan

HIGHLIGHTS

- PCN, a prototype rodent PXR activator ameliorated Con A-induced liver injury independent of PXR activation.
- PCN repressed the induction of chemokines at the early time point of Con A-induced liver injury.
- PCN diminished hepatic neutrophil infiltration induced by Con A challenge.

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ABSTRACT

The pregnane X receptor (PXR) is well-known as a key regulator of drug/xenobiotic clearance. Upon activation by ligand, PXR transcriptionally upregulates the expression of drug-metabolizing enzymes and drug transporters. Recent studies have revealed that PXR also plays a role in regulating immune/inflammatory responses. Specific PXR activators, including synthetic ligands and phytochemicals, have been shown to ameliorate chemically induced colitis in mice. In this study, we investigated an anti-inflammatory effect of pregnenolone 16α-carbonitrile (PCN), a prototypical activator for rodent PXR, in concanavalin A (Con A)-induced liver injury, a model of immune-mediated liver injury, using wild-type and *Pxr*$^{-/-}$ mice. Unexpectedly, pretreatment with PCN significantly ameliorated Con A-induced liver injury in not only wild-type but *Pxr*$^{-/-}$ mice as well, accompanied with lowered plasma ALT levels and histological improvements. Pretreatment with PCN was found to significantly repress the induction of *Cxcl2* and *Ccl2* mRNA expression and neutrophil infiltration into the liver of both wild-type and *Pxr*$^{-/-}$ mice at the early time point of Con A-induced liver injury. Our results indicate that PCN has unexpected immunosuppressive activity independent of PXR activation to protect mice from immune-mediated liver injury induced by Con A.

1. Introduction

Pregnane X receptor (PXR, NR1I2), an orphan member of the nuclear receptor superfamily, is well-characterized as a xenobiotic-sensing transcription factor (Kliewer et al., 2002). As PXR has a large and flexible ligand binding pocket, the receptor is able to respond to structurally diverse endobiotics and xenobiotics as ligands including steroids, therapeutics, and phytochemicals (Chang and Waxman, 2006). PXR is primarily expressed in the liver, small intestine, and colon. Upon activation, PXR plays roles in xenobiotic clearance and hepatic energy metabolism, by up- and

Abbreviations: PXR, pregnane X receptor; PCN, pregnenolone 16α-carbonitrile; Con A, concanavalin A; DSS, dextran sulfate sodium; TNF, tumor necrosis factor; LPS, lipopolysaccharide; NF-κB, nuclear factor κB; MPO, myeloperoxidase; SOCS1, suppressor of cytokine signaling; H&E, hematoxylin and eosin; qRT-PCR, quantitative reverse transcription-PCR.

* Corresponding author at: Laboratory of Pharmacotherapy of Life-Style Diseases, Graduate School of Pharmaceutical Sciences, Tohoku University, 6-3 Aramaki-Aoba, Aoba-ku, Sendai, Miyagi 980-8578 Japan.
E-mail address: kodama@m.tohoku.ac.jp (S. Kodama).

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down-regulating its target genes (Kliewer et al., 2002; Kodama and Negishi, 2013). In particular, PXR has been well-established as a master regulator of xenobiotic clearance. Activated PXR transcriptionally upregulates a battery of genes encoding drug-metabolizing enzymes and drug transporters.

Recent studies have revealed that PXR has a role in the regulation of immune system. Genetic association studies have implicated *PXR* as a gene significantly associated with the susceptibility to inflammatory bowel diseases (Dring et al., 2006; Langmann et al., 2004; Martinez et al., 2007). Immune-regulating activity of PXR has been demonstrated in the liver and intestinal cells which abundantly express PXR, employing both *in vivo* and *in vitro* models (Hu et al., 2010; Mencarelli et al., 2011; Shah et al., 2007). Treatment with pregnenolone 16α-carbonitrile (PCN), a prototypical activator for rodent PXR, ameliorated chemically induced colitis by dextran sulfate sodium (DSS) in wild-type mice but not in *Pxr*$^{-/-}$ mice (Shah et al., 2007). In primary cultures and cell lines, treatment with inflammatory stimuli such as tumor necrosis factor (TNF)-α and lipopolysaccharide (LPS) strongly upregulated the expression of a variety of immune-related genes, whereas cotreatment with PXR activators effectively downregulated their expression (Hu et al., 2010; Mencarelli et al., 2011). As a molecular mechanism underlying PXR-mediated immune regulation, an inhibitory cross-talk between PXR and nuclear factor κB (NF-κB), a master regulator of inflammatory response, has been implied, in which upon activation, PXR interferes with NF-κB signaling, resulting in the downregulation of NF-κB target genes such as *TNF-α*, *IL-1β*, and *NOS2*. However, the precise details are still not fully understood at the present time.

In addition to the liver and intestinal cells, PXR has been recently reported to be expressed in peripheral blood mononuclear cells (Dubrac et al., 2010). Immune activation increased the expression of PXR both in mouse and human T lymphocytes. In mouse models, treatment with PXR activators PCN and RU486 inhibited T lymphocyte proliferation and anergized T lymphocytes, in which PXR activated the expression of suppressor of cytokine signaling (SOCS1) prior to a downregulation of INF-γ production and inhibition of MEK1/2 and NF-κB signaling. However, it is still needed to clarify the precise role of PXR in immune cells.

Several commonly used clinical drugs that activate PXR have been known to have immunosuppressive effects (Zhou et al., 2009). For example, rifampicin, a prototypical ligand of human PXR, has long been known to act as an immunosuppressant in liver cells (Paunescu, 1970). In addition to typical PXR activators, a number of recent studies have assessed anti-inflammatory effects of various naturally occurring chemicals that effectively activate PXR, using chemically induced mouse models of colitis by DSS and trinitrobenzene sulfonic acid (Dou et al., 2013; Sepe et al., 2011; Zhang et al., 2015). Interestingly, ginsenosides have been demonstrated to inhibit the nuclear translocation (i.e. activation) of NF-κB in a PXR-dependent manner even though it is not PXR activator (Zhang et al., 2015).

In this study, we have addressed an anti-inflammatory effect of PCN, a prototypical activator for rodent PXR, in concanavalin A (Con A)-induced liver injury, a well described model of immune-mediated liver injury, using wild-type and *Pxr*$^{-/-}$ mice. Intravenous treatment with Con A, a T-cell mitogenic plant lectin, activates T lymphocytes, triggers the hepatic infiltration of leukocytes, and causes injury selectively in the liver (Bonder et al., 2004; Tiegs et al., 1992; Tsai et al., 2011). It has been demonstrated that neutrophils play a key role in the pathogenesis of Con A-induced liver injury, in which their depletion reduces T lymphocyte infiltration and ameliorates acute injury in the liver (Bonder et al., 2004; Hatada et al., 2005; Nakamura et al., 2001). Here we show the first evidence that PCN that is widely used to study the biological function of PXR in rodent models has an anti-inflammatory activity independent of PXR activation.

2. Materials and methods

2.1. Materials

PCN was purchased from Sigma-Aldrich (St. Louis, MO). Con A was obtained from Merck Millipore (Billerica, MA). Corn oil and Transaminase C II-test Wako were purchased from Wako Pure Chemical Industries (Osaka, Japan). Saline was purchased from Otsuka Pharmaceutical Factory (Naruto, Japan). Primers used for quantitative reverse transcription PCR (qRT-PCR) were commercially synthesized by Fasmac (Atsugi, Japan). All other reagents were from Sigma-Aldrich, Nakarai Tesque (Kyoto, Japan), or Wako Pure Chemical Industries.

2.2. Animal treatment

Male wild-type (C57BL/6, Charles River Japan, Yokohama, Japan) and *Pxr*$^{-/-}$ mice (kindly provided by Dr. Jeff Staudinger, University of Kansas, Lawrence, KS) were housed in a temperature-controlled environment with a 12 h-light/12 h-dark cycle with free access to food and tap water. All animal experiments were approved by the Institutional Animal Care and Use Committee at Tohoku University (Sendai, Japan) and performed in accordance with the Guidelines for Animal Experiments of Tohoku University (Sendai, Japan). Mice (8–10 weeks old) were randomly divided into two groups and were intraperitoneally treated with vehicle (corn oil, 20 ml/kg) or PCN (100 mg/kg). Three or six hours after treatment, corn oil-treated mice were divided into two groups. One group was intravenously administrated with vehicle (saline, 0.9% (w/v) sodium chloride, 12.5 ml/kg) and the other was intravenously treated with Con A (15 mg/kg). All mice pretreated with PCN were intravenously treated with Con A. For an experimental control of PCN treatment, mice were intraperitoneally treated with corn oil or PCN for 9 h. Mice were sacrificed by cervical dislocation at indicated time points, from which liver and spleen tissues were collected and used for analyses. At the time of sacrifice, blood samples were collected by tail bleed for plasma preparation. Plasma levels of alanine aminotransferase (ALT) were determined using Transaminase C-II test Wako.

2.3. qRT-PCR

Total RNAs were extracted from the tissues using Sepasol-RNA I (Nakarai Tesque), from which cDNAs were synthesized using High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Foster City, CA). qRT-PCR was performed with GoTaq qPCR Master Mix (Promega, Madison, WI) and specific primer sets for genes of interest in Thermal Cycler Dice Real Time System TP800 (Takara Bio, Otsu, Japan). The comparative Ct method was used for relative quantification of mRNA levels, normalized to those of *Gapdh* or 18S rRNA. The thermal cycle condition used was 95 °C for 2 min, followed by 40 cycles of 95 °C for 15 s and 60 °C for 1 min. Primer sets used for qRT-PCR are listed in supplemental Table 1.

2.4. Histology and immunohistochemistry

Liver tissues were fixed in 10% formalin neutral buffer solution (Wako Pure Chemicals Industries) and embedded in paraffin. Liver sections were stained with hematoxylin and eosin (H&E) or anti-myeloperoxidase (MPO) antibody following standard procedures by Morph Technology (Sapporo, Japan). MPO-positive cells were