Notoginsenoside R1 Attenuates Experimental Inflammatory Bowel Disease via Pregnane X Receptor Activation

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ABSTRACT

Notoginsenoside R1 (R1) is the main bioactive component in Panax notoginseng, an old herb medicine widely used in Asian countries in the treatment of microcirculatory diseases. However, little is known about the effect of R1 on inflammatory bowel disease (IBD). The present study demonstrated that R1 alleviated the severity of dextran sulfate sodium–induced colitis in mice by decreasing the activity of myeloperoxidase, the production of cytokines, the expression of proinflammatory genes, and the phosphorylation of IκB kinase, IκBα, and p65 in the colon. Further studies indicated that R1 dose-dependently activated human/mouse pregnane X receptor (PXR), a known target for decreasing inflammation in IBD, and upregulated the expression of genes involved in xenobiotic metabolism in colorectal cells and the colon. Ligand pocket–filling mutant (S247W/C284W or S247W/C284W/S208W) of the human PXR abrogated the effect of R1 on PXR activation. Time-resolved fluorescence resonance energy transfer PXR competitive binding assay confirmed R1 (ligand) binding affinity. In addition, PXR overexpression inhibited nuclear factor-κB (NF-κB)–luciferase activity, which was potentiated by R1 treatment. PXR knockdown by small interfering RNA demonstrated the necessity of PXR in R1–induced upregulation of the expression of xenobiotic-metabolizing enzymes and downregulation of NF-κB activity. Finally, the anti-inflammatory effect of R1 was confirmed in trinitrobenzene sulfonic acid–induced colitis in mice. These findings suggest that R1 attenuates experimental IBD possibly via the activation of intestinal PXR signaling.

Introduction

Inflammatory bowel disease (IBD), which includes ulcerative colitis (UC) and Crohn’s disease (CD), is a chronic inflammatory condition of the gastrointestinal tract. UC is limited to the colon, whereas CD can affect any part of the gastrointestinal tract from the mouth to the anus (Baumgart and Sandborn, 2007; Gupta et al., 2013). Conventional therapeutics for IBD have been associated with multiple adverse effects. New biologic therapies, including tumor necrosis factor-α (TNF-α) antagonists (e.g., infliximab, adalimumab, and golimumb) and integrin antagonists (e.g., vedolizumab and natalizumab), have profoundly influenced the management of IBD patients. However, there remain concerns about their potential side effects, tolerability, and high costs (Jobin, 2010; Gilroy and Allen, 2014).

Pregnane X receptor (PXR; NR1I2), one of the members of the nuclear receptor superfamily, is a xenobiotic/metabolic sensor that regulates the expression of drug-metabolizing enzymes and transporters involved in the clearance of many xenobiotic chemicals (Mani et al., 2013; Smutny et al., 2013). PXR has a bulky and flexible ligand-binding pocket that enables this receptor to accommodate structurally diverse ligands, including prescription drugs, natural products, dietary supplements, environmental pollutants, endogenous hormones, and bile acids (Zhang et al., 2008; Cheng et al., 2012). Ligand-activated PXR regulates the expression of a battery of genes, including phase I metabolic enzymes (CYP2B6, CYP2B9, CYP2C8, CYP2C9, CYP3A4, and CYP3A7), phase II metabolic enzymes [glutathione S-transferases (GSTs), UDP-glucuronosyltransferases (UGTs), and sulfotransfersases], and transporters [multidrug resistance protein 1 (MDR1), MDR2, multidrug resistance–associated protein 2 (MRP2), and the organic anion transporter polypeptide 2]. These enzymes and transporters are capable of recognizing a variety of

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ABBREVIATIONS: CD, Crohn’s disease; DSS, dextran sulfate sodium; GST, glutathione S-transferase; IBD, inflammatory bowel disease; iKK, IκB kinase; IL, interleukin; iNOS, inducible NO synthase; MCP-1, monocyte chemotactic protein-1; MDR, multidrug resistance protein; MPO, myeloperoxidase; MRP2, multidrug resistance–associated protein 2; NF-κB, nuclear factor-κB; PCN, pregnenolone-16α-carbonitrile; PCR, polymerase chain reaction; PXR, pregnane X receptor; R1, notoginsenoside R1; siRNA, small interfering RNA; SR12813, tetraethyl 2-(3,5-di-tert-buty1-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate; TNBS, trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor-α; TR-FRET, time-resolved fluorescence resonance energy transfer; UC, ulcerative colitis; UGT, UDP-glucuronosyltransferase.
xenobiotics to promote their clearance/detoxification, which is thought to be critical for maintaining intestinal barrier integrity (Zhang et al., 2008; Mencarelli et al., 2010). Indeed, disruption of the intestinal barrier function is linked to IBD. Gene analysis studies have indicated that the mRNA expression of PXR and MDR1 is significantly reduced in the colons of UC patients (Langmann et al., 2004). The present study and others have demonstrated that PXR activation inhibits the nuclear factor-κB (NF-κB) pathway and alleviates the severity of experimental IBD (Cheng et al., 2010; Dou et al., 2012). In a dextran sodium sulfate (DSS)–induced IBD model, administration of pregnenolone-16α-carbonitrile (PCN), a rodent-specific PXR ligand, attenuates development of colitis by decreasing the expression of NF-κB target genes [monocyte chemotactic protein-1 (MCP-1), inducible NO synthase (iNOS), interleukin (IL)-1β, IL-6, and TNF-α] and increasing the expression of phase II enzymes (GSTa1, GSTm1, and GSTt1) and transporters (MDR1a and MRP2) (Shah et al., 2007). Thus, targeted activation of PXR in recent years has become a therapeutic strategy for IBD (Cheng et al., 2010; Mencarelli et al., 2010).

Notoginsenoside R1 (R1) is a characteristic constituent of Radix notoginseng, a well-known herbal medicine widely used in Asian countries for treating microcirculatory disturbance-related diseases, such as cardiovascular disease, cerebral vascular disease, and liver dysfunction (Sun et al., 2007; Geng et al., 2010; Fan et al., 2012). R1 has multiple pharmacologic activities, including cardioprotective, neuroprotective, anti-inflammatory, and anticancer effects (Wang et al., 2009; Sun et al., 2013; Meng et al., 2014). However, thus far, no study has reported the effect of R1 on IBD. Therefore, in the current study, we used in vitro and in vivo models to investigate the effects of R1 on IBD and to uncover the possible underlying mechanisms mediated through PXR activation.

Fig. 1. R1 attenuated DSS-induced colitis in mice. (A) Changes in body weight following DSS induction of colitis. The data are plotted as a percentage of the original body weight. (B) The occurrence of bloody diarrhea. The data are plotted as a percentage of the total mice that had bloody diarrhea at different time points of DSS treatment. (C) Macroscopic observation of colon shortening. (D) Assessment of colon shortening. (E) Representative H&E-stained colon sections (magnification, 200×). (F) Histology scoring for H&E-stained colon sections. The data are expressed as the means ± S.D. of n = 10 mice in each group. *P < 0.05; **P < 0.01; ***P < 0.001 versus the DSS-treated group.
Materials and Methods

Cell lines, reagents, semiquantitative and real-time quantitative polymerase chain reaction (PCR), Western blot analysis, PXR-mediated NF-κB repression reporter assay, gene silencing, and time-resolved fluorescence resonance energy transfer (TR-FRET) assay details are mentioned in the Supplemental Methods.

Mice. Eight-week-old female C57BL/6 mice (20 ± 2 g) were obtained from the Shanghai Laboratory Animal Center (Shanghai, China) and were housed in cages at room temperature (25 ± 2°C) with a 12-hour light-dark cycle.

Table 1

<table>
<thead>
<tr>
<th>Group</th>
<th>DSS</th>
<th>TNBS</th>
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<tr>
<td></td>
<td>TNF-α</td>
<td>IL-6</td>
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<tr>
<td>Vehicle</td>
<td>14.6 ± 1.7</td>
<td>28.9 ± 2.3</td>
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<tr>
<td>R1</td>
<td>16.96 ± 1.1</td>
<td>23.79 ± 4.1</td>
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<tr>
<td>DSS/TNBS</td>
<td>127.4 ± 8.5</td>
<td>214.3 ± 17.9</td>
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<tr>
<td>DSS/TNBS + R1</td>
<td>61.5 ± 3.9**</td>
<td>164.2 ± 16.6*</td>
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*p < 0.05; **p < 0.01 versus DSS- or TNBS-treated group.

Fig. 2. The effects of R1 on NF-κB activation and target gene expression in vivo. (A) Total protein (40 μg) was extracted from colon samples (n = 6 per group) and examined for the expression of p-IκB-α, p-p65, p-1κB, 1κB, and β-actin by Western blot analysis. The results are representative of three independent experiments. (B) The levels of the expression of NF-κB target genes in colon samples isolated from mice (n = 6 per group) were determined using quantitative reverse-transcription PCR. The levels of expression are normalized to that of β-actin, and each bar represents the mean ± S.D. of two independent experiments performed using samples in triplicate. **P < 0.01; ***P < 0.001 versus Vehicle-treated group. #P < 0.05; ##P < 0.01 versus DSS-treated group. COX2, cyclooxygenase 2; ICAM-1, intercellular adhesion molecule-1; IFNγ, interferon-γ.
light/dark cycle. Standard mouse chow pellets and water were available ad libitum. The experiments were approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine.

**DSS and Trinitrobenzene Sulfonic Acid Colitis Models in Mice.** DSS or trinitrobenzene sulfonic acid (TNBS) colitis was induced in mice as described previously (Dou et al., 2012, 2013b). In brief, 4% (w/v) DSS (molecular mass 36–50 kDa; MP Biomedicals, Solon, OH) was administered in the drinking water (tap water) for 7 days, whereas control mice received tap water only (n = 10 mice in each group). In the TNBS model, food (but not water) was administered in the control group. Mice received a daily gavage of R1 (25 mg/kg) in 0.5% methylcellulose from day 1 to day 7. R1 dosage was selected based on the results of a previous report in which 25 mg/kg of R1 pretreatment blocked NF-κB activation and the subsequent myocardial inflammation and apoptotic responses in endotoxemic mice (Sun et al., 2013). Mice IBD studies were repeated again for independent confirmation of the initial data.

**Assessment of Colitis.** The severity of diarrhea was monitored throughout the experimental period. The total length of the colon was measured after sacrifice of the mice under anesthesia. The distal colon was fixed in 10% buffered formalin, embedded in paraffin, and processed for routine HE staining of sections. Histologic scoring was performed in a blinded fashion by two pathologists to obtain a combined score of inflammatory cell infiltration (score 0–3) and tissue damage (score 0–3), as described previously (Dou et al., 2013b).

**Wild-Type/Mutant PXR Transactivation Reporter Assays.** HT-29 cells (1 × 10⁶) in 100 μl of transfection buffer (Cell Line Nucleofector Kit V; Lonza AG, Walkersville, MD) were electroporated using the program W-017 according to the manufacturer’s recommendation (Lonza Nucleofector II instrument; Amnax Biosystems, Gaithersburg, MD). For the PXR wild-type transactivation assay, the cells were transfected with 1 μg of CYP3A4-luciferase reporter combined with 0.1 μg of pRL-TK, and 0.5 μg of the plasmid expressing wild-type human PXR (pSg5-hPXR) or wild-type mouse PXR (pSg5-mPXR), as shown in schematic diagrams (see Fig. 3B). For the PXR mutant transactivation assay, the cells were transfected with 1 μg of MRP2-luciferase reporter combined with 0.1 μg of pRL-TK, and 0.5 μg of plasmid expressing the wild-type hPXR or the hPXR double mutant (S247W/C284W) or the hPXR triple mutant (S247W/C284W/S208W), as shown in schematic diagrams (see Fig. 6A). For detailed plasmid information, refer to our previous reports (Wang et al., 2008; Venkatesh et al., 2011). For the wild-type PXR transactivation assay, the cells were treated with R1 (0, 1, 10, or 25 μM), rifampicin (10 μM, for hPXR transactivation), or PCN (10 μM, for mPXR transactivation) for 24 hours. For mutant PXR transactivation assay, the cells were treated with R1 (0 or 25 μM) or rifampicin (10 μM) for 24 hours. The luciferase activity of the cell extracts was measured and expressed as the fold induction compared with that of the control cells, as previously described (Dou et al., 2012).

**Measurement of Cytokines.** Colon segments were homogenized in ice-cold phosphate-buffered saline. The homogenates were centrifuged at 3000g for 10 minutes, and the supernatants were assayed for the determination of levels of TNF-α and IL-6, as described previously (Dou et al., 2013a). The level of each cytokine was evaluated using enzyme-linked immunosorbent assay kits according to the manufacturer’s protocols (R&D Systems, Minneapolis, MN), and the results are expressed in picograms per milligram of protein in each sample.

**Myeloperoxidase Assay.** Tissue myeloperoxidase (MPO) activity, which is linearly related to neutrophil infiltration in inflamed tissue, was assayed to monitor the degree of inflammation. MPO activity in colon tissues was measured as previously described (Zhang et al., 2014), and the values were expressed as units per milligram of protein.

**Statistical Analysis.** All of the data are expressed as means ± S.D. The differences between the groups were analyzed using one-way analysis of variance followed by the least significant difference post-hoc test. The statistical analyses were performed using the SPSS 16.0 software package (SPSS, Chicago, IL). P < 0.05 was considered statistically significant.

Fig. 3. The effects of R1 on NF-κB target gene expression in vitro and on PXR activation. (A) The levels of the mRNA expression of NF-κB target genes in lipopolysaccharide (LPS)-stimulated RAW264.7 macrophages following R1 treatment (0 and 25 μM, for 48 hours) were detected by semiquantitative reverse-transcription PCR. The results are representative of three independent experiments. (B) Wild-type human/mouse PXR transactivation reporter assay. Transient transactivation assays were performed in HT-29 cells cotransfected with CYP3A4-reporter, pRL-TK, and pSg5-hPXR or pSg5-mouse PXR (mPXR). The cells were incubated with R1 (0, 1, 10, or 25 μM), rifampicin (Rif, 10 μM, for hPXR transactivation), or PCN (10 μM, for mPXR transactivation) for 24 hours. A standard dual-luciferase assay of the cell lysates was performed, and the results are expressed as the fold induction compared with that of the control cells. *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle-treated cells. COX2, cyclooxygenase 2; iCAM-1, intercellular adhesion molecule-1; IFNγ, interferon-γ.
Results

**R1 Ameliorated DSS-Induced Colitis.** The DSS-induced colitis model is a well-established chemical IBD model with clinical features resembling human UC (Neurath and Travis, 2012). R1 treatment significantly ameliorated the DSS-induced loss of body weight, bloody diarrhea, colon shortening, and histologic damage (Fig. 1). The activity of MPO, as well as the levels of TNF-α and IL-6, was markedly reduced by R1 treatment (Table 1). In addition, none of the mice that received R1 alone exhibited loss of body weight, diarrhea, colon shortening, or mucosal disruption at any point during the study.

**R1 Inhibited NF-κB Activation and Downregulated NF-κB Target Gene Expression.** Intestinal NF-κB activation plays a central role in the pathogenesis of IBD (Atreya et al., 2008). Major steps for NF-κB activation involve the activation of IκB kinase (IKK)-α/β, which is followed by IκBα degradation and the subsequent p65/p50 nuclear translocation (Tak and Firestein, 2001). Using Western blot analysis, we observed a phosphorylation/activation of IKK-α/β and p65 in the colonic tissue of DSS-treated mice (Fig. 2A). IκBα phosphorylation and degradation were induced in mice with DSS-induced colitis. In contrast, administration of R1 obviously reduced the expression of phosphorylated p65 and phosphorylated IKK-α/β in DSS-induced colitis, and the phosphorylation/degradation of IκBα was also repressed. To determine the effect of R1 on the expression of NF-κB signaling genes, several proinflammatory cytokines and chemokines in the colon were evaluated using quantitative reverse-transcription PCR analysis. The increase in the levels of inflammatory mediators (intercellular adhesion molecule-1, cyclooxygenase-2, TNF-α, interferon-γ, iNOS, MCP-1, IL-1α, IL-1β, IL-2, and IL-6) after DSS treatment dramatically decreased in the mice that received R1 treatment (Fig. 2B).

Next, we investigated whether R1 affects the expression of NF-κB target genes in RAW264.7 mouse macrophage cells using semiquantitative reverse-transcription PCR. The RAW264.7 macrophage cell line is a well-established model system for inflammatory studies (Wu et al., 2011). In accordance with in vivo data, R1 treatment obviously decreased the expression of iNOS, intercellular adhesion molecule-1, MCP-1, cyclooxygenase-2, interferon-γ, TNF-α, and IL-15 in lipopolysaccharide-stimulated RAW264.7 cells (Fig. 3A).

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**Fig. 4.** Role for PXR in R1-mediated NF-κB luciferase (Luc) inhibition. (A) HT-29 cells were electroporated with the pGL4.32[luc2P/NF-κB-RE/Hygro] reporter combined with pRL-TK and pSG5-hPXR or pSG5-mock. PXR overexpression was verified by Western blot analysis (left). After transfection, the cells were treated with TNF-α (20 ng/ml) alone or in combination with R1 (25 μM) for 24 hours. A standard dual-luciferase activity of the cell lysates was measured, and the results are expressed as the fold induction compared with that of the control cells (designated as 1). The results are presented as the means ± S.D. of three independent experiments. *P < 0.05 versus hPXR-transfected cells treated with TNF-α alone. (B) The human PXR gene was silenced by PXR siRNA transfection in the aforementioned HT-29 cells previously transfected with the pGL4.32[luc2P/NF-κB-RE/Hygro] reporter and the human PXR expression plasmid pSG5-hPXR. The depletion of PXR by siRNA transfection in HT-29 cells was verified using Western blot (left). After transfection, the cells were treated with TNF-α (20 ng/ml) alone or cotreated with R1 (25 μM) for 24 hours. A standard dual-luciferase assay of the cell lysates was performed. The results are presented as the means ± S.D. of three independent experiments. *P < 0.05 versus control siRNA transfection samples treated with TNF-α alone.
R1 Activated Both Human and Mouse Wild-Type PXR. In transient transfection gene reporter assays, R1 increased human or mouse PXR-mediated CYP3A4 promoter transcription in a concentration-dependent manner (Fig. 3B). In addition, rifampicin and PCN significantly activated human and mouse PXR, respectively.

R1 Inhibited NF-κB Activity in a PXR-Dependent Manner. To determine whether activation of PXR plays a direct role in the NF-κB repression by R1, a transient transfection reporter assay was performed. HT-29 cells have low or undetectable levels of constitutive human PXR expression (Dou et al., 2014). In cells transfected with the NF-κB–luciferase reporter and the PXR mock, treatment with the known NF-κB pathway activator TNF-α led to increased NF-κB activity; however, R1 had no effect on NF-κB activity in the absence of human PXR expression (Fig. 4A). In cells transfected with the NF-κB–luciferase reporter and the human PXR expression vector, TNF-α–stimulated NF-κB activity was significantly inhibited by R1 in the presence of human PXR expression. To further demonstrate that PXR is specifically involved in the suppression of NF-κB by R1, PXR small interfering RNA (siRNA) transfection was conducted in the aforementioned HT-29 cells, which had been previously transfected with the NF-κB–luciferase reporter and the human PXR expression plasmid. As expected, the regulatory effect of R1 on TNF-α–stimulated NF-κB activity was abrogated (Fig. 4B).

Fig. 5. The role of PXR in the R1-mediated upregulation of PXR target genes. Quantitative reverse-transcription PCR was performed to assess the mRNA expression of CYP3A11 (CYP3A4 for human), UGT1A1, and MDR1a in colon samples isolated from mice (n = 6 per group) orally fed 25 mg/kg R1 for 7 days (A) or in LS174T cells (n = 3) treated with 25 μM R1 for 48 hours (B). The levels of expression are normalized to that of β-actin, and each bar represents the mean ± S.D. of two independent experiments performed using samples in triplicate. *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle-treated group. (C) The depletion of PXR in LS174T cells using siRNA transfection was verified using Western blot (top). The mRNA expression of UGT1A1 and MDR1a was assessed using quantitative reverse-transcription PCR (bottom). The results are expressed as the means ± S.D. from triplicate samples of two independent experiments. ***P < 0.001 versus vehicle-treated cells transfected with control siRNA.
R1 Upregulated the Expression of Xenobiotic-Metabolizing Genes In Vitro and In Vivo. PXR target genes are critical components in the intestinal barrier function against xenobiotics and bacteria (Langmann et al., 2004; Mencarelli et al., 2010). In the current study, we observed a robust upregulation of the expression of CYP3A11, UGT1A1, and MDR1a in the colons of mice that received 7 days of R1 treatment (Fig. 5A). Consistent with the in vivo results, an increase in the expression of CYP3A4, UGT1A1, and MDR1a was observed in LS174T cells exposed to R1 treatment (Fig. 5B).

R1 Upregulated the Expression of Xenobiotic-Metabolizing Genes in a PXR-Dependent Manner. To explore the mechanistic involvement of PXR in the modulation of the genes responsible for xenobiotic metabolism, the human PXR gene expression in LS174T colon adenocarcinoma cells was reduced by PXR siRNA transfection. As shown in Fig. 5C, the treatment of LS174T cells with an anti-PXR siRNA significantly decreased the expression of PXR. In addition, the regulatory effect of R1 on the mRNA expression of UGT1A1 and MDR1a was abrogated in cells transfected with anti-PXR siRNA.

Ligand Pocket–Filling Mutants of Human PXR Abolished R1-Mediated PXR Activation. To provide evidence that R1 indeed binds to the ligand-binding pocket of the human PXR and subsequently activates the transcription of MR2 promoter, we performed a transient transactivation reporter assay using the double-mutant (S247W/C284W) and triple-mutant (S247W/C284W/S208W) constructs of the human PXR ligand-binding pocket. The results showed that the double or triple mutants of PXR led to ligand-independent constitutive activation of the MR2 promoter (Fig. 6A), which is consistent with the results of previous reports (Wang et al., 2008; Venkatesh et al., 2011). In addition, both rifampicin and R1 activated the wild-type human PXR, whereas none of them activated the double or triple mutant.

Confirmation of the PXR-Binding Properties by TR-FRET Assay. To confirm the direct binding of R1 to the ligand-binding pocket of the human PXR, a LanthaScreen TR-FRET PXR competitive binding assay (Life Technologies, Grand Island, NY) was performed. R1 concentration-dependently decreased the TR-FRET emission ratio (Fig. 6B). In addition,

![Fig. 6. Effects of R1 on the transactivation of PXR mutants and TR-FRET assay. (A) Transient transcription assays were performed in HT-29 cells cotransfected with MRP2-luciferase reporter, pRL-TK, and the wild-type hPXR (pSG5-hPXR) or the hPXR double mutant (S247W/C284W) or the hPXR triple mutant (S247W/C284W/S208W). The cells were treated with R1 (0 and 25 \( \mu M \)) or rifampicin (Rif; 10 \( \mu M \)) for 24 hours. Luciferase activities were measured, and the results are expressed as the fold induction compared with that of the control cells. The results are presented as the means ± S.D. of three independent experiments. **P < 0.01; ***P < 0.001 versus vehicle-treated cells. (B) The LanthaScreen TR-FRET competitive binding assay was used to evaluate the ability of R1 (0, 12.5, 25, 50, and 100 \( \mu M \)) to interact with PXR in vitro. Rifampicin (10 \( \mu M \)) and SR12813 (SR; 1 \( \mu M \)) were included as positive control PXR ligands. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm by that at 495 nm. The results are expressed as the means ± S.D. from a representative experiment performed in quadruplicate. *P < 0.05; **P < 0.01; ***P < 0.001 versus vehicle-treated cells. RXR, retinoid X receptor.](http://jpet.aspetjournals.org/)


both rifampicin and SR12813 tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate) significantly decreased the TR-FRET ratio (Fig. 6B).

**R1 Treatment Attenuated the Severity of TNBS-Induced Colitis.** Next, to determine whether R1 also had a therapeutic effect on another chemical model of colitis, we tested its effects on some parameters of colitis induced by the hapten TNBS, which constitutes a CD model (Neurath and Travis, 2012). The rectal administration of TNBS led to ulcers in the rectum, increased MPO activity, and upregulated TNF-α and IL-6 levels (Fig. 7; Table 1). Oral R1 administration significantly improved these parameters and ameliorated the histologic pathology. No significant changes were observed in vehicle or R1-treated normal mice.

**Discussion**

Chemically-induced colitis models have been developed and extensively used to elucidate the pathogenic mechanisms of IBD. One of the most widely used experimental models is developed by treating animals with DSS in drinking water for 6–10 days. The resulting inflammation generally affects the mucosal lining of the intestinal wall, with disease features that resemble human UC (Neurath and Travis, 2012). In the current study, colitis was induced by DSS treatment, and the exposure of mice to R1 ameliorated the disease hallmarks, such as body weight loss, bloody diarrhea, colon shortening, and histologic damage. To assess whether R1 would have similar effects on another chemical model of colitis, we tested its effects on some parameters of colitis induced by TNBS, which constitutes a CD model of transmucosal inflammation (Neurath and Travis, 2012). The results showed that the rectal administration of TNBS dissolved in ethanol induced severe colitis in mice, and that R1 treatment reduced the extent of histologic damage and colon shortening. Since no report about the effect of R1 on UC or CD has been available, our findings are unique in establishing the role of R1 in ameliorating the UC- or CD-like features in murine models of IBD. Notably, none of the mice that received R1 alone exhibited apparent body weight loss, diarrhea, colon shortening, or mucosal disruption throughout this study, indicating the relative safety of the R1 treatment.

Recent studies have shown that PXR is involved in the pathogenesis of IBD (Cheng et al., 2010, 2012). Loss of PXR function has been associated with intestinal inflammation in animal models (Cheng et al., 2010; Dou et al., 2012). Gene expression profiling of the colon tissues from UC and CD patients demonstrated a significant inhibition of PXR and its target genes (Shah et al., 2007). Several polymorphisms in the PXR locus have been proved to be associated with an increased susceptibility to IBD (Langmann et al., 2004; Dring et al., 2006). PXR has a bulky and flexible ligand-binding pocket, which enables it to accommodate a structurally promiscuous library of ligands (Tolson and Wang, 2010). Upon activation, PXR heterodimerizes with retinoid X receptor and binds to the response elements of PXR target genes. In the present study, we found that R1 dose-dependently activated human PXR, which is likely due to binding to the ligand-binding domain of PXR. It has long been known that ligand-activated PXR regulates the expression of biotransformation enzymes and transport proteins involved in the metabolism and elimination of harmful substances from the body (Langmann et al., 2004).

**Fig. 7.** R1 attenuated TNBS-induced colitis in mice. (A) Macroscopic observation and assessment of colon shortening (n = 10). (B) Representative H&E-stained colon sections (magnification, 200×) and histology scores. The results are expressed as the means ± S.D. of n = 10 mice in each group. *P < 0.05; **P < 0.01 versus TNBS-treated group.
The detoxification properties of PXR and its target genes are necessary to maintain the integrity of the intestinal epithelial barrier (Shah et al., 2007). In this study, we found that R1 upregulated the expression of CYP3A, UGT1A1, and MDR1a, and this effect was mediated by PXR. It was recently reported that the natural compound curcumin decreases the susceptibility of MDR1a−/− mice to spontaneous colonic inflammation through PXR activation (Nones et al., 2009). Our results support the notion that enhanced intestinal epithelial integrity via PXR-mediated induction of xenobiotic detoxification might contribute to the observed anti-inflammatory effects of R1, but further studies are required.

Although the exact etiology of IBD remains unknown, it has been generally accepted that the increase of proinflammatory cytokines, such as IL-1β and TNF-α, within colonic tissues plays an important role in the pathogenesis of IBD (Shah et al., 2007). Also, it has been demonstrated that all of these proinflammatory cytokine genes are transcriptionally regulated by NF-κB (Tak and Firestein, 2001). NF-κB can be activated by diverse stimuli, including proinflammatory cytokines, microbes and microbial products, and oxidative stress (Liu and Wang, 2011). Activation of NF-κB leads to the gene expression of proinflammatory cytokines and chemokines involved in the pathogenesis of IBD. The most abundant form of NF-κB in cells is the p50/p65 heterodimer, which is normally sequestered in the cytoplasm by binding to the inhibitory protein IκBα. Following various stimuli, IκBα is rapidly phosphorylated by IKK, ubiquitinated, and subsequently degraded by proteasome, allowing NF-κB to translocate to the nucleus, where it drives the expression of its target genes (Zhou et al., 2006). Following various stimuli, IκBα is rapidly phosphorylated by IKK, ubiquitinated, and subsequently degraded by proteasome, allowing NF-κB to translocate to the nucleus, where it drives the expression of its target genes (Zhou et al., 2006).

In our study, we found that R1 treatment not only decreased the proteins of phosphorylated IκBα/α, phosphorylated IκBα, and phosphorylated p65, but also downregulated the proinflammatory gene expression, and reduced the activity of MPO and the accumulation of TNF-α and IL-6. Further studies indicated that R1 decreased the activity of NF-κB in a PXR-dependent manner. In fact, recent molecular and pharmacologic studies have revealed a mutual suppression between PXR and the NF-κB signaling pathway (Xie and Tian, 2006; Wahli, 2008). High NF-κB activity inhibits the activation of PXR (Gu et al., 2006), whereas PXR activation suppresses the activity of NF-κB and the expression of its target genes (Zhou et al., 2006).

IBD is associated with a considerable reduction in the quality of life of patients, and currently no curative treatment is available (van der Marel et al., 2011; Gupta et al., 2013). The use of medicinal plants or their active components is becoming an area of our ongoing screening program to evaluate the anti-inflammatory potentials of natural compounds (Dou et al., 2013a; Zhang et al., 2014), we investigated the anti-inflammatory effects of R1 on DSS- or TNBS-induced colitis. To our knowledge, the present study is the first to demonstrate a beneficial effect of R1 on chemically induced IBD (UC- or CD-like). The anti-inflammatory effect of R1 might be associated with the activation of PXR signaling. The results support further evaluation of the therapeutic potential of R1 in human IBD.

Authorship Contributions

Participated in research design: Z. Wang, Dou.
Conducted experiments: Zhang, Ding, Ren, Deng.

Contributed new reagents or analytic tools: B. Wang, Wei.

Performed data analysis: Zhang, Sun.

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References


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Notoginsenoside R1 Attenuates Experimental Inflammatory Bowel Disease via Pregnane X Receptor Activation

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This file includes: Supplemental Methods, including cell lines, reagents, semi-quantitative and real-time quantitative polymerase chain reaction (qPCR), western blot analysis, PXR-mediated NF-κB repression reporter assay, gene silencing, and TR-FRET assay protocols.
Supplemental Methods

Cell Lines and Reagents. The HT-29 and LS174T human colon adenocarcinoma cell lines and the RAW264.7 mouse macrophage cell line were obtained from the American Type Culture Collection (ATCC) and cultured according to ATCC recommendations. Notoginsenoside R1 (3β,6α,12β)-20-(β-D-Glucopyranosyloxy)-3,12-dihydroxydammamcar-24-en-6-yl2-O-β-D-xylopyranosyl-β-D-glucopyranoside, C_{47}H_{80}O_{18}, molecular weight (MW) 933.13, high-performance liquid chromatography (≥ 98%) was kindly provided by the Shanghai R&D Center for the Standardization of Traditional Chinese Medicine, Shanghai, China. Dextran sulfate sodium (DSS) (MW 36000-50000) was acquired from MP Biochemical LLC (Solon, OH). Rifampicin (3-4-Methylpiperazinyliminomethyl, C_{43}H_{58}N_{4}O_{12}, MW 822.94), pregnenolone-16α-carbonitrile (5-pregnenolone-16a-carbonitrile, PCN, C_{22}H_{31}NO_{2}, MW 341.49), SR12813 (Tetraethyl 2-(3,5-di-tert-butyl-4-hydroxyphenyl)ethenyl-1,1-bisphosphonate, C_{24}H_{42}O_{7}P_{2} , MW 504.53), trinitrobenzene sulfonic acid (TNBS), lipopolysaccharide (LPS), donkey serum, paraformaldehyde, methylcellulose, formalin, Tween-20, ethanol and DMSO were obtained from Sigma-Aldrich (St. Louis, MO). The NF-κB reporter vector pGL4.32[luc2P/NF-κB-RE/Hygro] and a dual-luciferase reporter assay system were purchased from Promega (Madison, WI). SYBR Premix ExTaq Mix was obtained from Takara Bio Inc. (Otsu, Japan). A LanthaScreen™ TR-FRET PXR competitive binding assay system, a SuperScript III first-strand synthesis system, Fluor 488-conjugated anti-rabbit IgG (A-21206), Triton X-100, TRIZol, and DAPI reagent were obtained from Invitrogen (Carlsbad, CA). BSA and protease inhibitor cocktail tablets were purchased from Roche Diagnostics (Mannheim, Germany). The PXR siRNA duplex (sc-44057), the control siRNA duplex (sc-37007) and the horseradish peroxidase-conjugated secondary antibodies were obtained from Santa Cruz Biotechnology (Santa Cruz, CA). The human PXR antibody (ab85451) was obtained from Abcam (Cambridge, MA). The TNF-α reagent and mouse antibodies directed against p-IKK-α/β (#2078), p-p65 (#3033), p-IκBα (#2859), IκBα (#4812) and β-actin (#4970) were purchased from Cell
Signaling Technology (Danvers, MA). Mouse TNF-α and IL-6 ELISA kits were obtained from R&D systems (Minneapolis, MN). An MPO activity assay kit was purchased from CytoStore (Calgary, AB, Canada). Agarose, ethidium bromide, RIPA lysis buffer and enhanced chemiluminescence (ECL) western blot detection reagents were purchased from Thermo Scientific (Waltham, MA).

**Western Blot Analysis.** Cells or colon tissues in RIPA lysis buffer with fresh protease inhibitor cocktail tablets were disrupted by incubation for 30 minutes on ice or by homogenization on ice, respectively, and then collected supernatants by spinning for 10 minutes at 10,000 g, at 4°C. Equal amounts of protein (40 µg) were separated by 10% SDS-PAGE and transferred to nitrocellulose membranes. Antibodies directed against PXR (1:1000), p-IKK-α/β (1:1000), p-p65 (1:1000), p-IκBα (1:1000), IκBα (1:1000 and β-actin (1:2000) were used as recommended by the manufacturers. The membranes were incubated with horseradish peroxidase (HRP)-conjugated secondary antibodies (anti-rabbit or anti-mouse) and developed using ECL detection reagents. The protein bands were quantified by the average ratios of integral optic density (IOD) following normalization to the housekeeping gene β-actin expression.

**Semi-quantitative and Real-time Quantitative (q) Polymerase Chain Reaction (PCR).** RAW 264.7 macrophages were cotreated with R1 (25 µM) and LPS (2 µg/ml) for 48 h. RNA was extracted using TRIzol reagent. 3 µg of total RNA was converted to first-strand cDNA using a SuperScript III reverse transcriptase kit. The primer sequences used in semi-quantitative PCR amplification are as follows: 5’-ACATTCAAGATCCCGAAGGC-3’/5’-TTTGATGTCACGCACGATTT-3’ for miNOS, 5’-TGATGGCAGCCCTCTATGTT-3’/5’-AATGAAGTCAGCGTTTCTTGG-3’ for mICAM-1, 5’-AAGTTGACCGCTAAATCTGA-3’/5’-TGAAAGGGAATACCATAACA-3’ for mMCP-1, 5’-GAAGTCTTTGGTCTGGTGCCT-3’/5’-CTGCTGGTTTGGAATAGTTGCT-3’
for mCOX2,
5'-GCTCTGAGACAATGAACGC-3'/'5'-TTTCTCCCACATCTATGCACT-3'
for mIFNr,
5'-CTGTGAAGGGAATGGGTGTT-3'/'5'-CAGGGAAGAATCTGGGAAAGGTC-3'
for mTNF-α,
5'-CAGAATGGGAGGTTGGATGTC-3'/'5'-AAGAGTGGGTGACAGAAGG-3'
for mIL-15, and
5'-TGCTGTCCCTGTATGCTCT-3'/'5'-TTTGATGTCACGCACGATTT-3' for mβ-actin. The DNA thermal cycler conditions used were 94°C for 5 min (pre-denature), and 35 cycles of 94°C for 1 min, annealing at 58°C for 30 s and extension at 72°C for 45 s, followed by a final extension of 72°C for 2 min. PCR products were separated by electrophoresis on 2% agarose gels and stained with ethidium bromide. The amount of target gene was normalized with beta-actin as the internal control gene. The primer sequences used in qPCR amplification are as follows:
5'-GATGAAAGAAAGTCGCTCG-3'/'5'-GCTGGACATCAGGGTGAGT-3' for hCYP3A4,
5'-AATAGTTGTCCCTAGCCTGAC-3'/'5'-TCTTTCACATCCTCCCTTTG-3' for hUGT1A1,
5'-AGCCCATCCTGCTTGACTGC-3'/'5'-TGTATGTTGGCCTCCTTTGC-3' for hMDR1a,
5'-GGAAATCGTGACAGTTAC-3'/'5'-TCAGGCACGTGGTACCCT-3' for hβ-actin,
5'-TGGAGATGGAATACCTGGAT-3'/'5'-GAATCATCACTGTTGACC-3' for mCYP3A11,
5'-GCACGAAGTTGTGGCACTGC-3'/'5'-GAATCATCAGCTCGTAGCTCTT-3' for mUGT1A1,
5'-TGTGATTGCGTTTGGAGGAC-3'/'5'-CCATACCAGAATGCCAGAGC-3' for mMDR1a,
5'-GGGAATCTTGGAGGAGTTG-3'/'5'-GTGAGGGCTTGGCTGAGTG-3' for
miNOS,
5'-CGCTGTGCTTTGAGAACTGT-3'/5'-AGGTCCTTGCCTACTTGCTG-3' for
mICAM-1,
5'-AAGTTGACCCGTAATCTGA-3'/5'-TGAAAGGGAATACCATAACA-3' for
mMCP-1,
5'-GAAGTCTTTGTCTGGGTGCTCT-3'/5'-GCTCCTGCTTGAGTATGTCG-3' for
mCOX2,
5'-CGTGGGAACCTGCGAAGAGG-3'/5'-AGACAGAAAGCGTGTTGCGC-3' for
mTNF-α,
5'-AGCAACAACATAAGCGTCAT-3'/5'-CCTCAAACTTGGCAATACTC-3' for
mIFNr,
5'-GTTCTGCCATTGACCATCTC-3'/5'-TGATACTGTCACCCGGCTCT-3' for
mIL-1α,
5'-GGCTGGACTGTTTCTAAATGC-3'/5'-ATGGTTTCTTTGTGACCCTGTA-3' for
mIL-1β,
5'-TCAGCAACTGTGGGTGACTT-3'/5'-AGTGATTAGCAAGGGTGAGA-3' for
mIL-2, 5'-ATGGCAATTCTGATTGTATG-3'/5'-GACTCTGGCTTTGTCTTCT-3' for
mIL-6, and
5'-CAGCCTTCCCTTCTTGAT-3'/5'-TGGCATAGAGGTCTTTACGG-3' for
mß-actin. PCR reactions were carried out using SYBR Premix ExTaq Mix in an ABI
Prism 7900 real-time PCR System (Life Technologies, Carlsbad, CA). The thermal
cycler parameters were as follows: 1 cycle of 95°C for 30 s, then 40 cycles of
denaturation (95°C, 5 s) and combined annealing/extension (60°C, 30 s). Gene
expression changes were calculated by the comparative Ct method, and the values
were normalized to the internal ß-actin control.

**PXR-mediated NF-κB Repression Reporter Assay.** 2×10⁶ HT-29 cells in 100 µl
transfection buffer (Cell Line Nucleofector Kit V) were co-electroporated with 1 µg
pGL4.32[ luc2P/NF-κB-RE/Hygro] luciferase reporter vector (Promega, Madison, WI),
0.5 µg expression vector (pSG5-hPXR or pSG5 control) and 0.1 µg pRL-TK vector
using Lonza Nucleofector II instrument (program Q-009). The cells were transferred to 48-well plate following transfection. After overnight culture, the cells were treated with TNF-α (20 ng/ml) alone or in combination with R1 (0 or 25 μM) for 24 h. The cells were harvested in passive lysis buffer (Promega) and luciferase activity was detected using the dual-luciferase reporter assay system (Promega). Luminescence was detected by Turner Bio-systems Luminometer 20/20n. Normalization and calculations of fold induction were performed as previously published (Dou et al. 2013).

**Gene Silencing.** 2×10^6 LS174T or HT-29 cells in 100 μl transfection buffer (Cell Line Nucleofector Kit V) were electroporated with PXR siRNA targeting human PXR mRNA using program T-020 (Lonza Nucleofector II instrument). A non-targeting siRNA was used as a negative control. The cells were seeded in 6-well plates following transfection and cultured overnight. For LS174T cells, the cells were treated with or without R1 (25 μM) for 48 h and were harvested for qRT-PCR or western blot studies. For HT-29 cells, which had been previously transfected with the NF-κB-luciferase reporter and the human PXR expression plasmid (pSG5-PXR), the cells were treated with TNF-α (20 ng/ml) alone or in conjunction with R1 (25 μM) for 48 h following transfection. The luciferase activity was measured and the results were expressed as the fold induction compared with that of the control cells.

**Time-resolved Fluorescence Resonance Energy Transfer (TR-FRET) Assay.** The human PXR binding assay was performed using the Lanthascreen™ TR-FRET PXR competitive binding assay system (Life Technologies, NY) as described previously (Venkatesh, et al., 2011), in which a test compound competes and displaces a reference fluorescent-labeled ligand from the recombinant terbium-labeled PXR-LBD. Briefly, 10 μl of test compounds (final concentration, 0, 12.5, 25, 50 and 100 μM) was placed in quadruplicate into the wells of a black, round-bottomed 384-well assay plate. Next, 5 μl of 4× Fluormone PXR (SXR) Green was added to each well, followed by 5 μl of 4× PXR-LBD (GST)/DTT/4×Tb anti-GST antibody. The plate was gently rocked and then incubated in the dark at room temperature for 1
h. TR-FRET was measured using an EnVision® Multilabel Plate Reader (PerkinElmer, Boston, MA) at an excitation wavelength of 340 nm and at emission wavelengths of 520 and 495 nm. The TR-FRET ratio was calculated by dividing the emission signal at 520 nm by that at 495 nm. Rifampicin (final concentration, 10 µM) and SR12813 (final concentration, 1 µM) was included as positive control of PXR ligands.

References