Ginsenosides Regulate PXR/NF-κB Signaling and Attenuate Dextran Sulfate Sodium–Induced Colitis

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ABSTRACT

Pregnane X receptor (PXR) activation exhibits anti-inflammatory effects via repressing nuclear factor-κB (NF-κB); however, its overactivation may disrupt homeostasis of various enzymes and transporters. Here we found that ginsenosides restore PXR/NF-κB signaling in inflamed conditions without disrupting PXR function in normal conditions. The effects and mechanisms of ginsenosides in regulating PXR/NF-κB signals were determined both in vitro and in vivo. Ginsenosides significantly inhibited NF-κB activation and restored the expression of PXR target genes in tumor necrosis factor-α–stimulated LS174T cells. Despite not being PXR agonists, ginsenosides repressed NF-κB activation in a PXR-dependent manner. Ginsenosides significantly increased the physical association between PXR and the NF-κB p65 subunit and thereby decreased the nuclear translocation of p65. Ginsenoside Rb1 and compound K (CK) were major bioactive compounds in the regulating PXR/NF-κB signaling. Consistently, ginsenosides significantly attenuated dextran sulfate sodium–induced experimental colitis, which was associated with restored PXR/NF-κB signaling. This study indicates that ginsenosides may elicit anti-inflammatory effects via targeting PXR/NF-κB interaction without disrupting PXR function in healthy conditions. Ginsenoside Rb1 and CK may serve as leading compounds in the discovery of new drugs that target PXR/NF-κB interaction in therapy for inflammatory bowel disease.

Introduction

Pregnane X receptor (PXR) is a ligand-activated transcription factor and a master regulator of drug transport and metabolism (Cheng et al., 2012). PXR is expressed predominantly in the liver and intestine of mammals. It is activated by a range of structurally diverse xenobiotics, such as the antibiotic rifampicin (RIF), the herbal antidepressant St. John’s wort, endogenous hormones, and bile acids (Ma et al., 2008). On activation, PXR forms a heterodimer with retinoid X receptor that binds to specific PXR response elements on the promoters of target genes, such as cytochrome P450 3A (CYP3A), multidrug resistance protein 1 (MDR1), and multidrug resistance protein 2. In addition to the well-validated functions in regulating transporters and drug-metabolizing enzymes, PXR plays important roles in the pathologic development of a panel of diseases, including cancer, diabetes, and inflammatory bowel diseases (IBD). Of particular interest, reciprocal repression between PXR and nuclear factor-κB (NF-κB) has been described (Zhou et al., 2006). The expression of PXR and several of its target genes has been shown to be suppressed in the colon of patients with IBD (Langmann et al., 2004; Blokzijl et al., 2007), suggesting that PXR may play a critical role in IBD.

NF-κB is a key transcription factor that can modulate several steps in the inflammatory cascade by inducing the expression of proinflammatory genes (Hayden and Ghosh, 2004; Perkins and Gilmore, 2006; Karrasch and Jobin, 2008). Excessive production of proinflammatory cytokines, such as tumor necrosis factor α (TNF-α), interleukin-1β (IL-1β), IL-6, and interferons, was detected in IBD (Sartor, 1994; Ishiguro, 1999.) The activated NF-κB p65 subunit (NF-κB) p65; IKK, IκB kinase; IκB; SB, sodium butyrate; TPA, phorbol-12-myristate-13-acetate; TBS, Tris-buffered saline; TNF-α, tumor necrosis factor α.

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ABBREVIATIONS: CK, compound K; COX, cyclooxygenase; DAI, disease activity index; DSS, dextran sulfate sodium; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; GSE, ginseng saponin extract; IBD, inflammatory bowel diseases; IL-1β, interleukin-1β; IκB, inhibitor of NF-κB; IKK, IκB kinase; INOS, inducible nitric oxide synthase; MDR, multidrug resistance; MPO, myeloperoxidase; NF-κB, nuclear factor-κB; PBS, phosphate-buffered saline; PVDF, polyvinylidene fluoride; PXR, pregnane X receptor; RIF, rifampicin; RT-PCR, reverse-transcription quantitative real-time polymerase chain reaction; siRNA, small interfering RNA; TBST, Tris-buffered saline containing 0.5% Tween-20; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF-α, tumor necrosis factor α.
repressive relationship, it is reasonable to expect that restoration of the balance between PXR and NF-κB would be a promising strategy for the therapy of IBD, a disease with complicated causes and poor therapeutic means (Zhou et al., 2014).

Ginseng, the root of *Panax ginseng*, has been widely used as a food product and medicinal ingredient in Asian countries for more than 2000 years (Jia and Zhao, 2009; Kang et al., 2011). Ginsenosides are considered the major active ingredients of ginseng (Ouyang et al., 2014; Zhao et al., 2014). Ginseng saponins have various pharmacologic effects, such as antioxidant, antiinflammatory activities, and neuroprotective effects (Attelle et al., 1999; Radad et al., 2006; Kim et al., 2010). A recent investigation suggested that ginseng suppressed key inflammatory players such as cyclooxygenase (COX)-2, inducible nitric oxide synthase (iNOS), and NF-κB (Park et al., 2009; Kim et al., 2012). Ginsenoside compound K (CK) was shown to promote recovery of DSS-induced colitis by suppressing NF-κB activation (Li et al., 2014). Ginsenoside Rd can attenuate 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis via modulating mitogen-activated protein kinase signaling pathways (Yang et al., 2012b). Ginseng has also been suggested as an anti-inflammatory agent or adjuvant treatment of the patients of IBD (Hosfeth and Wargovich, 2007).

Although the anti-inflammatory effects of ginsenosides and its remedies effects against IBD have been well reported, the molecular mechanisms remain established (Yang et al., 2012a; Li et al., 2014; Ye et al., 2014). Here we hypothesized that ginsenosides may elicit its anti-inflammatory effects in IBD via restoring PXR/NF-κB signaling in the inflamed colon. Unexpectedly, we found that ginsenosides, without the influence of PXR activity in the normal conditions, significantly combat NF-κB signaling in a PXR-dependent manner.

### Materials and Methods

#### Chemicals and Reagents.
Ginseng saponin extract (GSE; ginsenoside Rb1, Rb2, Rc, Rd, Re, Rg1, Rg3, Rh1, Rh2, CK, protopanaxadiol (PPD), and protopanaxatriol (PPT) were purchased from Hongjiu Biotech Co. Ltd. (Jilin, China). The contents of ginsenosides (Rb1 11.2%, Rb2 11.1%, Rc 10.5%, Rd 7.7%, Re 8.9%, Rf 0.9%, Rg1 3.3%, Rg2 1.4%, and Rh1 0.2%) were determined using a validated liquid chromatography-mass spectrometry method in our laboratory (Hao et al., 2010). Rifampicin (RIF) was purchased from Sigma-Aldrich (Shanghai, China). Dextran sulfate (DSS) was provided by MP Biomedicals (Solon, OH) (mol. wt. 36–50 kDa). TNF-α (human, recombinant) was purchased from Promega Corporation (Madison, WI). PXR small interfering RNA (siRNA) and control siRNA were purchased from Invitrogen Trading Shanghai Co. (Invitrogen Life Technologies, Shanghai, China).

#### Animals and Treatment.
Male C57BL/6 mice (8 weeks old, 20–22 g) were obtained from the Animal Ethics Committee of China Pharmaceutical University. Mice were divided into four groups: normal, GSE, DSS, and DSS + GSE. Acute colitis was induced by administration of 3% (w/v) DSS in drinking water for 5 days. GSE at a dose of 400 mg/kg of body weight was administered via oral gavage 3 days before the DSS treatment of a consecutive 9 days. The dose for GSE treatment was designed on the basis of previous reports (Wong et al., 2010; Jang et al., 2012) and the results from a preliminary experiment.

#### Samples Collection and Histologic Evaluation.
After the mice were killed under anesthesia, the colons were immediately removed and fixed in 10% formalin, paraffin-embedded, sectioned, and stained with H&E. Histologic score of H&E-stained specimens of the colon was determined by two pathologists in a blinded fashion. Histologic sections were scored using a validated scoring system as described previously (Williams et al., 2001; Wen et al., 2014).

#### Cell Culture.
The human colon cancer cell line LS174T was obtained from Shanghai Cell Resource Center (Shanghai Institutes for Biologic Sciences, China) and cultivated at 37°C in an atmosphere of 95% air and 5% CO2 with Dulbecco’s modified Eagle’s medium supplemented with 10% fetal bovine serum (Hyclone, Logan, Utah), 100 U/ml penicillin, 100 μg/ml streptomycin, and 20 mM t-glutamine. The cell medium was changed every other day. Cells were passaged on reaching ~80% confluence.

#### Real-Time Quantitative Reverse Transcriptase PCR.
Total RNA was extracted by using Trizol reagent (TaKaRa Biotechnology Co., Ltd., Dalian, China). The content of RNA was estimated by measuring the absorbance at 260 nm. Purified total RNA was reverse-transcribed using the PrimerScript RT Reagent Kit (TaKaRa Biotechnology Co., Ltd.) according to the manufacturer’s protocol. Primer sequences are as follows (forward 5’-3’, reverse 5’-3’): mouse IL-1β (CCT CGT GCT GTC GGA CCC AT, CCC AAG CAG CAG AGA CAC G), mouse Cox-2 (TGA GCA ACT ATT CCA AAC CAG C, CCA GCT AGT CTT CCA TCA CTA C), mouse iNOS (CTT GCT GTC AGA ACT GTG, ACC TCC TGT CCT ACG TGT TGA GAG AC, CCA TAC CAG AAT GCC AGA GC), mouse glucuronidase-3-phosphate dehydrogenase (Gapdh) (CAG CTT CAA CAG CAA CTA CTA C, GGA GTA TAC TTC ATC ATC ATC ATC), human NF-κB (AAC CTC CTC GCC TTC ATC AA, GAG AGA CCC CTC CCA GAT AG), human COX-2 (TGA GCA TAC AGT TGT TGC, TGG TGT TGC AGA ACT GC), human iNOS (CTT GCT GTC AGA ACT GTG, AGG AGC TCC TGT CCT ACG TGT), mouse PXR (GTG CAA CAG CTT CAC AA, TCC TGG AGG CCA CCA TGA GG), mouse Cyp3aa1 (CCG AGG GTT TTT TGT TCA TCA, CAG CTT CAT CAG TCT CAT CC), mouse Mdr1a (TTG GTG TGC TGT TGC AGG AC, CCA TAC CAG AAT GCC AGA GC), mouse glucuronyltransferase.

### Western Blot Analysis.
Colon tissues were disrupted by homogenization on ice and centrifuged at 4°C (12,000g, 15 minutes). The supernatants were collected, and the protein concentrations were determined by BCA assay. Protein lysates were loaded onto 10% SDS-polyacrylamide gels. Peroxidase-labeled rabbit anti-mouse IgG secondary antibodies were purchased from Cell Signaling Technology. The antibodies used were as follows: anti-NF-κB p65 (no. 8242, Cell Signaling Technology, Danvers, MA), anti-NF-κB phospho-p65 (no. 3033, Cell Signaling Technology), anti-PXR (sc-48403, Santa Cruz Biotechnology, Santa Cruz, CA), and anti-β-actin (A5441, Sigma-Aldrich, St. Louis Park, MN). The horseradish peroxidase-conjugated goat anti-mouse/ rabbit IgG secondary antibodies were purchased from Cell Signaling Technology.

#### RNA Interference Assay.
LS174T cells were seeded into six-well plates, incubated at 37°C, and transfected 24 hours after reaching 70% confluence.

Cells were transiently transfected with PXR siRNA targeting the human PXR mRNA. Control siRNA, a nontargeting siRNA, was used as a negative control. After transfection, cells were incubated with GSE (100 μg/ml), Rb1 (10 μM), CK (10 μM), or RIF (10 μM) for 24 hours, followed by an additional incubation with or without TNF-α (20 ng/ml) for 6 hours. At the end of the incubation, cells were rinsed, scraped, and used in RT-PCR or Western blot analysis as described herein.

**Immunohistochemistry.** Tissue specimens were fixed in 10% formalin for 24 hours, dehydrated, and paraffin-embedded. Standard immunohistochemical procedures were performed. Tissue sections were incubated with the primary antibody (rabbit monoclonal anti-NF-κB p65, rabbit polyclonal anti-PXR) for 2 hours at room temperature, followed by incubation with the prediluted horseradish peroxidase-conjugated secondary antibody for 30 minutes. For negative controls, 1% of nonimmune serum in phosphate-buffered saline (PBS) replaced the primary antibodies. The immunohistochemistry staining of NF-κB p65, and PXR was scored by measuring the integrated optical density of at least three visions of each slice using Image Pro-plus 6.0 software (Media Cybernetics, Inc., Rockville, VA). Data are expressed as mean ± S.E.M. of six mice.

**Immunofluorescence Staining.** LS174T cells were seeded in cell imaging dish (Eppendorf, Germany). After overnight incubation, cells were treated with GSE (100 μg/ml), Rb1 (10 μM), or CK (10 μM) for 3 hours, followed by an additional incubation with or without TNF-α (20 ng/ml) for 6 hours. At the end of the incubation, cells were harvested and fixed with 4% paraformaldehyde solution at 20°C for 20 minutes. After washing in PBS, cells were permeabilized with 0.2% Triton X-100 in PBS at room temperature for 5 minutes. After incubation in blocking buffer containing 0.1% Triton X-100 and 5% bovine serum albumin, cells were incubated with rabbit NF-κB p65 antibody (no. 8242, Cell Signaling Technology) at 4°C overnight and then with Alexa Fluor 488-conjugated anti-rabbit IgG antibody (A-21206, Invitrogen) at room temperature for 30 minutes in 1% bovine serum albumin in PBS. Fluorescence photographs were obtained using a Zeiss 710 confocal microscope (Zeiss, Germany).

**Comnunoprecipitation.** A communoprecipitation assay was conducted to examine the physical association between PXR and NF-κB p65 subunits. Briefly, cells were collected and lysed in NP-40 buffer and sonicated. After centrifuging the cell lysate for 10 minutes at 15,000g, the clear lysate was immunoprecipitated with PXR (sc-48403) antibody or an irrelevant rabbit IgG as a negative control, together with protein A-agarose (Invitrogen, Carlsbad, CA). The immunoprecipitates were washed three times with washing buffer, resuspended in 4× sample buffer (Bio-Rad), and heated for 5 minutes at 100°C. After a spin-down, equal amounts of protein from the supernatants were electrophoresed on 10% SDS-PAGE gels, transferred onto PVDF membranes, and immunoblotted with an antibody against PXR and NF-κB p65, and PXR was scored by measuring the integrated optical density of at least three visions of each slice using Image Pro-plus 6.0 software (Media Cybernetics, Inc., Rockville, VA). Data are expressed as mean ± S.E.M. of six mice.

**Ginsenosides Regulate PXR/NF-κB Signaling in Colonic Cell Lines.** To determine the effect of GSE in regulating PXR/NF-κB signaling, the mRNA levels of multiple PXR and NF-κB target genes were determined in LS174T cells in both basal and TNF-α-stimulated conditions. In the basal condition, the typical human PXR agonist RIF significantly increased mRNA expression of PXR target genes CY3A4 and MDR1 in LS174T cells, whereas GSE had little effect; however, both GSE and RIF significantly counteracted TNFα-induced reduction of the mRNA levels of PXR, CY3A4, and MDR1 in LS174T cells (Supplemental Fig. 1, A–C). Likewise, both GSE and RIF significantly reduced TNFα-induced upregulation of IL-1β, NF-κB, and iNOS (Supplemental Fig. 1, D–F). TNFα treatment to LS174T cells for 30 hours caused a significant reduction of PXR protein levels and increase in the ratio of phosphorylated to total NF-κB p65, both of which were abolished by GSE (Supplemental Fig. 1G). These results suggest that GSE is able to recover the imbalanced PXR/NF-κB signaling on TNFα stimulation, although GSE is not a PXR agonist.

**PXR Silencing Abrogates the Anti-inflammatory Activity of GSE.** Our results suggest that GSE can restore PXR/NF-κB signaling without the activation of PXR. Previous studies indicated that PXR and NF-κB are mutally repressed. We thus asked whether the effects of GSE in attenuating NF-κB activation were PXR dependent. To this end, we silenced the PXR gene using siRNA in human colon carcinoma LS174T cells and determined the effects of GSE in NF-κB activation. With scrambled siRNA treatment, GSE and RIF significantly attenuated TNFα-induced activation of NF-κB; the mRNA levels of CY3A4, MDR1, IL-1β, and iNOS were largely recovered; however, the PXR siRNA treatment resulted in a significant reduction of the protein levels of PXR (Supplemental Fig. 2A) and largely abrogated the effect of GSE and RIF in attenuating TNFα-induced activation of NF-κB (Supplemental Fig. 2, B–F). In agreement, GSE significantly repressed p-p65 accumulation without the influence of the total p65 levels, which was markedly abrogated by PXR silencing. These results indicate that the effects of GSE in combating TNFα-induced activation of NF-κB are largely mediated by PXR.

**Screening for Anti-inflammatory Compounds in GSE.** GSE is a mixture containing dozens of ginseng saponin compounds. We thus sought to screen for the dominant compounds contributing to the anti-inflammatory activity of GSE. Using the mRNA level of iNOS as an index, we screened a panel of 12 compounds and found that ginsenoside Rb1 and CK are the compounds with the most potent anti-inflammatory activity in GSE (Supplemental Fig. 3). Thereafter, we focused on evaluating the effects of ginsenoside Rb1 and CK on PXR/NF-κB signaling. The concentration for ginsenoside Rb1 (10 μM) and CK (10 μM) was optimized from a preliminary study to ensure sufficient anti-inflammatory activity and without apparent cytotoxicity. Ginsenoside Rb1 and CK significantly reduced TNFα–induced upregulation of IL-1β and iNOS mRNA levels (Fig. 1, A, B, E, and F), and restored the mRNA levels of PXR and CY3A4 in LS174T cells (Fig. 1, C, D, G, and H). TNFα caused a significant reduction in PXR protein levels and increase in the ratio of phosphorylated to total NF-κB p65, both of which were significantly abrogated by ginsenoside Rb1 and CK (Fig. 1).

**Anti-inflammatory Activity of Ginsenoside Rb1 and CK Is PXR Dependent.** Because GSE elicits its anti-inflammatory activity in a PXR-dependent manner, we asked whether this also holds true for ginsenoside Rb1 and CK. To this end, the anti-inflammatory activity of ginsenoside Rb1 and CK was determined in LS174T cells transfected with negative control or PXR siRNA. As expected, the knockdown of PXR protein levels by PXR siRNA almost completely abrogated the anti-inflammatory activity of ginsenoside Rb1 and CK. In PXR silenced LS174T cells, the treatment of ginsenoside Rb1 and CK could not counteract TNFα-induced upregulation of IL-1β and iNOS, and downregulation of CY3A4 (Fig. 2, A–F). Furthermore, PXR silencing obviously abolished the effect of ginsenoside Rb1 and CK on counteracting TNFα-induced increase in the ratio of phosphorylated to total NF-κB p65 (Fig. 2, G and H).

To further validate the role of PXR in the anti-inflammatory activity of ginsenosides, we performed a confocal microscopy analysis of p65 nuclear translocation in LS174T cells transfected with negative control or PXR siRNA (Fig. 2J). The nuclear levels of p65 translocation were increased in the LS174T cells treated with TNF-α, which was largely abolished by GSE, ginsenoside Rb1, or CK. Silence of PXR almost completely abrogated the effect of GSE, ginsenoside Rb1, and CK on counteracting TNFα–stimulated p65 nuclear translocation. All these results support that the effects of ginsenosides in combating TNFα–triggered inflammation are largely PXR dependent.

**Ginsenosides Promote PXR and NF-κB p65 Interaction.** Ginsenosides have little effect in PXR function in the basal condition;
Fig. 1. Effects of ginsenoside Rb1 and CK on PXR and NF-κB signaling in vitro. The mRNA levels of (A, E) PXR, (B, F) CYP3A4, (C, G) IL-1β, and (D, H) iNOS were determined using RT-PCR in LS174T cells. (I) Phospho-p65, p65, and PXR protein levels were determined by Western blot analysis. (J) The relative density of the protein levels was performed by densitometric analysis of the blots. After overnight incubation, LS174T cells were treated with ginsenoside Rb1 (10 μM), CK (10 μM), or RIF (10 μM) for 24 hours, followed by an additional incubation with or without TNF-α (20 ng/ml) for 6 hours (Fig. 1A-H) or 36 hours (I and J). At the end of the incubation, cells
However, their anti-inflammatory activities are largely PXR-dependent. These results prompted us to hypothesize that ginsenosides might influence the physical association between PXR and NF-κB p65 subunit (Mencarelli et al., 2011). To this end, we performed a coimmunoprecipitation assay using anti-PXR antibody. Because the long-duration treatment (36 hours) of TNF-α significantly reduced

were harvested and analyzed by RT-PCR or Western blot. Data are expressed as mean ± S.E.M. of triplicates of two independent experiments. *P < 0.05, **P < 0.01 versus vehicle-treated wells; #P < 0.05, ##P < 0.01 versus TNF-α-treated wells.

Fig. 2. PXR is an important determinant in repressing NF-κB signaling by ginsenoside Rb1 and CK. The mRNA levels of (A, D) CYP3A4, (B, E) IL-1β, and (C, F) iNOS were determined using RT-PCR in LS174T cells. (G, H) Phospho-p65 and p65 protein levels were determined by Western blot analysis. (I) The relative density of the protein levels was performed by densitometric analysis of the blots. (J) NF-κB nuclear translocation was detected by immunofluorescence analysis using p65 antibody. LS174T cells were transfected with scrambled siRNA or PXR siRNA. After incubation for 48 hours, cells were treated with ginsenoside Rb1 (10 μM), CK (10 μM), or Rif (10 μM) for 24 hours, followed by an additional incubation with or without TNF-α (20 ng/ml). At the end of the incubation, cells were harvested and analyzed by RT-PCR or Western blot. Data are expressed as mean ± S.E.M. of triplicates of two independent experiments. *P < 0.05, **P < 0.01 versus vehicle-treated wells; #P < 0.05, ##P < 0.01 versus TNF-α-treated wells.
the protein levels of PXR that might make it difficult to evaluate the physical interaction between PXR and NF-κB, a protocol of shorter duration of treatment (6 hours) was applied in the coimmunoprecipitation assay. As shown in Fig. 3, GSE, ginsenoside Rb1, and CK, without the influence of the total PXR levels, increased the degree of the physical association between PXR and NF-κB p65 in TNF-α-stimulated LS174T cells. This result indicates that ginsenosides may combat TNF-α-stimulated inflammatory responses via promoting the physical interaction between PXR and NF-κB p65 subunit.

**GSE Ameliorates DSS-Induced Colitis.** Several previous reports indicated that several isolated ginsenoside compounds including ginsenoside Rd and Rh2 (Yang et al., 2012a,b; Ye et al., 2014) are effective against experimental colitis. It remains unclear, however, whether GSE, which has been widely used by human beings as a food supplement, is also effective against the pathologic development of colitis. To answer this question, we extended this study to validate the effect of GSE against DSS-induced colitis. DSS induced a significant loss of body weight, whereas GSE treatment dramatically ameliorated DSS-induced body weight loss (Fig. 4A). GSE treatment also significantly combated DSS-induced colon shortening (Fig. 4B). Diarrhea symptoms appeared after day 5; the mice with GSE treatment showed less diarrhea and bloody diarrhea than did the DSS group (Fig. 4C). Histologic studies showed that GSE administration to DSS mice resulted in significant protection of the colon crypt structures and less severe histologic inflammation (Fig. 4, D and E). Additionally, GSE administration significantly attenuated DSS-triggered activation of myeloperoxidase, a biochemical marker of lipid peroxidation (Fig. 4F).

**GSE Recovers PXR/NF-κB Signaling in Inflamed Colon.** We extended to determine whether the anti-inflammatory effect of GSE was associated with the recovery of PXR/NF-κB balance. The results showed that the mRNA levels of IL-1β, IL-6, Cox-2, and iNOS, all of which are typical NF-κB target genes, were remarkably induced in the colonic tissue of DSS mice. In contrast, the increase in these inflammatory mediators after DSS administration was obviously retarded in the mice with GSE treatment (Fig. 5, A–D). Moreover, Western blotting analysis showed that DSS-induced phosphorylation of NF-κB p65 was markedly decreased by GSE (Fig. 5H). GSE had little effect on PXR activation in healthy mice, which is in line with the results from in vitro study; however, GSE significantly attenuated DSS-induced reduction in the mRNA levels of PXR and its target genes (Fig. 5, E–G). Immunohistochemical analysis showed that NF-κB p65 was significantly reduced, whereas PXR increased by GSE treatment compared with DSS group (Fig. 5, I and K).

**Discussion**

PXR and NF-κB were mutually repressed. Thereafter, the activation of PXR by several agonists has been found effective in combating against IBD (Shah et al., 2007; Dou et al., 2012). In this study, we found that the compounds of ginsenosides, which are not PXR agonists themselves, repressed NF-κB-activated inflammatory responses in a PXR-dependent manner via enforcing the interaction between PXR and p65. In cultured colon cancer cell lines, the treatment with ginsenosides markedly inhibited TNF-α-induced NF-κB activation and restored the expression of PXR and its target genes CYP3A4 and MDR1 in a PXR-dependent manner. Ginsenosides have little effect, however, on the activation of PXR in normal cells. The treatment of ginsenosides significantly attenuated DSS-induced colitis, which was associated with the recovery of the balance between PXR and NF-κB. Our study suggests that ginsenosides may selectively regulate the function of PXR in inflamed tissues via targeting PXR/ NF-κB interaction without the influence of PXR activation in healthy conditions.

The anti-inflammatory activities of ginseng extract and its isolated compounds of ginsenosides have been widely confirmed in various in vitro and in vivo conditions; however, the molecular mechanisms remain largely unclear. Ginseng extract could block NF-κB activity in various cell types, including microglial cells and human breast cancer cells (Park et al., 2009; Peralta et al., 2009). In line with these findings, we found that GSE markedly repressed TNF-α-stimulated NF-κB activation in LS174T cells. An interesting finding was that the expression of PXR and its target genes CYP3A4 and MDR1 was significantly recovered with GSE treatment; however, unlike the typical PXR agonist RIF, GSE has little effect in PXR function in the basal condition of LS174T cells. A previous report also indicated that ginseng had marginal effect in the activation of PXR (Mooiman et al., 2013). These results suggest that the restored expression and activation of PXR on GSE treatment is probably via a direct inhibition of NF-κB signaling; however, the silencing of PXR largely abrogated the anti-inflammatory activity of GSE in LS174T cells, supporting a PXR-dependent effect in inhibiting NF-κB signaling by GSE.

To further validate this finding, we screened the isolated single compounds of ginsenosides in GSE with anti-inflammatory activity. The screening results showed that ginsenoside Rb1, a dominant compound in GSE, possesses the strongest anti-inflammatory activity among the ginsenosides screened. Additionally, we found that CK, a major metabolite of ginsenosides in the gastrointestinal tract, on degradation by gut microbiota also possesses strong anti-inflammatory activity. In accordance with the finding of GSE, both ginsenoside Rb1 and CK inhibit NF-κB signaling in a PXR-dependent manner, although both compounds affect little of the basal PXR function. Previous studies indicated that NF-κB p65 directly interacted with the DNA-binding domain of retinoid X receptor α, an important partner for PXR transactivation, providing a mechanistic explanation to the mutual repression between PXR and NF-κB p65. This mechanism might not explain the PXR-dependent effect of ginsenosides in repressing NF-κB signaling, however, because ginsenosides are not PXR agonists. To this end, we tested whether PXR could directly interact with NF-κB p65. The coimmunoprecipitation test supports that

![Fig. 3. A coimmunoprecipitation assay of the physical association between PXR and NF-κB p65 subunit. The cell lysates were immunoprecipitated with PXR antibody and an irrelevant rabbit IgG as a negative control, together with protein A-agarose. The immunoprecipitates were then electrophoresed on SDS-PAGE gels, transferred onto PVDF membranes, and immunoblotted with an antibody against PXR and NF-κB p65. Similar results were observed in three independent experiments.](attachment:image)
PXR directly interacts with p65 and that ginsenosides can markedly promote this interaction. In addition, the confocal analysis of p65 nuclear translocation confirmed that ginsenosides could decrease p65 nuclear translocation in a PXR-dependent manner. All these results suggest that PXR might physically associate with p65 in the cytoplasm, thereby preventing p65 nuclear translocation, which is a basic step for NF-κB transactivation of its downstream proinflammatory genes. It seems that ginsenosides can strengthen the physical interaction between PXR and p65, thus showing PXR-dependent anti-inflammatory effects.

In addition, it is important to note that TNF-α treatment significantly reduces the mRNA and protein levels of PXR, which was recovered on GSE, as well as ginsenoside Rb1 and CK treatment. Similar results were observed in the colon of DSS-treated mice. It is reasonable to assume that the restored expression of PXR may contribute to repress NF-κB activation. These results suggest that ginsenosides may inhibit NF-κB transactivation and downstream inflammation via the restoration of PXR expression and the enforcement of the physical interaction between PXR and NF-κB.

PXR is well known for its central role in the regulation of drug metabolism and is a major regulator of CYP3A gene expression in both human and rodents (Cheng et al., 2012). CYP3A is a member of the cytochrome P450 monooxygenase superfamily, which is responsible for the oxidative metabolism of numerous drugs and toxicants (Goodwin et al., 2002). PXR expression and several of its target genes have been shown to be suppressed in the colon of patients with IBD (Langmann et al., 2004; Blokzijl et al., 2007). Humans with genetic variation in the PXR encoding gene were associated with a decrease in PXR activity and an increase in susceptibility to IBD (Dring et al., 2004).

**Fig. 4.** GSE attenuates DSS-induced colitis in mice. (A) Body weight changes after DSS induction of colitis. Data are plotted as percentage of basal body weight. (B) Colon length. (C) Representative H&E-stained colon sections (magnification 200×). (D) Histology score. (E) Disease activity index. (F) Myeloperoxidase (MPO) activity (U/g tissue). Values are expressed as mean ± S.E.M. of six to eight mice in each group.*P < 0.05, **P < 0.01 versus normal; #P < 0.05, ##P < 0.01 versus DSS model.
In the current study, we showed that GSE significantly attenuated DSS-induced colitis, which is associated with repressed NF-κB activity and restored PXR and CYP3A mRNA expression in the inflamed colon. Although it has previously been reported that Panax ginseng influence CYP3A activity (Malati et al., 2012), we confirmed that ginsenosides had no effect on Pxr and Cyp3a11 mRNA expression in the healthy mice, which is consistent with the findings from in vitro LS174T cells and the results from other groups (Gurley et al., 2002; Anderson et al., 2003).

Ginseng and the ginsenosides extracts are widely available as food supplements. The current study suggests that the consumption of GSE...
may be of benefit to IBD patients. Moreover, we showed that ginsenoside Rb1 and its metabolite CK may serve as useful leading compounds for the development of drugs targeting PXR/NK-kβ interaction against IBD. Because the strong activation of PXR may arouse undesirable effects, such as the disrupted expression of various kinds of drug-metabolizing enzymes and transporters, the strategy of developing PXR agonists to anti-inflammatory drugs remains to be established. In contrast, the current study indicates that targeting PXR/ NK-kβ interaction may be an alternative strategy for the development of anti-inflammatory drugs without disrupting PXR function in normal tissues.

**Authorship Contributions**

**Conducted experiments:** Zhang, Yan, Xie, Y. Wu, Zhao, M. Mu, Wu

**Contributed new reagents or analytic tools:** Cao, Cheng, L. Wang, Zhu

**Performed data analysis:** Zhang, Hao, H. Wang

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**References**


