Paeoniflorin abrogates DSS-induced colitis via a TLR4-dependent pathway

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1Shanghai Key Laboratory of Complex Prescription and MOE Key Laboratory for Standardization of Chinese Medicines, Institute of Chinese Materia Medica, Shanghai University of Traditional Chinese Medicine, Shanghai, China; 2Department of Pharmacognosy, China Pharmaceutical University, Nanjing, China; 3Departments of Medicine and Genetics, Albert Einstein College of Medicine, New York, New York; and 4Shanghai R&D Center for Standardization of Traditional Chinese Medicine, Shanghai, China

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Zhang J, Dou W, Zhang E, Sun A, Ding L, Wei X, Chou G, Mani S, Wang Z. Paeoniflorin abrogates DSS-induced colitis via a TLR4-dependent pathway. Am J Physiol Gastrointest Liver Physiol 306: G27–G36, 2014. First published November 14, 2013; doi:10.1152/ajpgi.00465.2012.—Paeonia lactiflora Pall is one of the most well-known herbs in China, Korea, and Japan for more than 1,200 years. Paeoniflorin, the major bioactive component of peony root, has recently been reported to have anti-inflammatory activity. However, the underlying molecular mechanism is unclear. The present study was to explore the possible mechanism of paeoniflorin in attenuating dextran sulfate sodium (DSS)-induced colitis. Pre- and coadministration of paeoniflorin significantly reduced the severity of colitis and resulted in downregulation of several inflammatory parameters in the colon, including the activity of myeloperoxidase (MPO), the levels of inflammatory cytokines (MCP-1, Cox2, IFN-γ, TNF-α, IL-6, and IL-17). The decline in the activation of NF-κB p65, ERK, JNK, and p38 MAPK correlated with a decrease in mucosal Toll-like receptor 4 (TLR4) but not TLR2 or TLR5 expression. In accordance with the in vivo results, paeoniflorin downregulated TLR4 expression, blocked nuclear translocation of NF-κB p65, and reduced the production of IL-6 in LPS-stimulated mouse macrophage RAW264.7 cells. Transient transfection assay performed in LPS-stimulated human colon cancer HT-29 cells indicated that paeoniflorin inhibits NF-κB transcriptional activity in a dose-dependent manner. TLR4 knockdown and overexpression experiments demonstrated a requirement for TLR4 in paeoniflorin-mediated downregulation of inflammatory cytokines. Thus, for the first time, the present study indicates that paeoniflorin abrogates DSS-induced colitis via decreasing the expression of TLR4 and suppressing the activation of NF-κB and MAPK pathways.

DSS-induced colitis; TLR4; NF-κB; MAPK; paeoniflorin

INTESTINAL EPITHELIUM REPRESENTS the first defense in preventing pathogenic invasion of commensal bacteria. It provides a critical interface between microorganisms and their hosts (1, 33). To maintain healthy homeostasis of intestine, mammals have evolved a defensive network called innate and adaptive immune defensive systems (9, 31). The innate immunity plays a central role in regulating immune responses to intestinal pathogen-derived microorganisms by recognizing the presence of specific bacterial antigens through an extensive family of pattern recognition receptors (PRRs), which are situated in intestinal epithelial cells and recognize conserved microbial molecules, called pathogen-associated molecular patterns (1, 31). Toll-like receptor 4 (TLR4) is a PRR that has been found in Drosophila melanogaster and mammals, and compelling research has shown that lipopolysaccharide (LPS), which is the major component of the outer membrane of gram-negative bacteria, binds to TLR4 and triggers signaling cascades mediated by myeloid differentiation factor MyD88 to activate the transcription factor NF-κB and eventually results in inflammatory response (14, 27). There is increasing evidence that LPS is not the only ligand for TLR4. Heparan sulfate, the extra-domain-A of fibronectin, hyaluronic acid, and fibrinogen, have signal through TLR4 (8). TLR4 is strongly upregulated in inflammatory bowel disease (IBD) patients as well as in acute dextran sulfate sodium (DSS)-induced colitis mice, implying that changes in TLR4 expression and subsequent alterations in the innate immune response contribute to the pathogenesis of IBD (14, 16).

Recent studies indicated that Lactobacillus suntoryeus, a gut commensal, blocks inflammatory mediators (Cox2, TNF-α, IL-1, and IL-6) through suppression of TLR4-mediated NF-κB activation in mice with TNBS-induced colitis (19). Lubbad et al. (23) have reported that amelioration of TNBS-induce colitis by curcumin is mediated through TLR4-linked NF-κB inhibition. We recently demonstrated that naringenin ameliorates DSS-induced colitis via transsecting TLR4/NF-κB signaling cascades (4). In addition, TLR4 is indicated to be linked with MAPK pathway in IBD. TLR4 mononclonal antibody (TLR4mAb) ameliorates DSS-induced colitis via suppression of p38 MAPK pathway (21). TLR4 ligand LPS caused significant phosphorylation (activation) of MAPK in intestinal mucosal enterochromaffin cells isolated from Crohn’s colitis patients (18). Thus it is proposed that many therapeutic agents that abrogate intestinal inflammation might transsect with the TLR4 signaling pathway (7, 34).

Paeonia lactiflora Pall is an ornamental and medicinal plant. A decoction of the dried root of peony has been used in the treatment of rheumatoid arthritis in China for more than 1,200 years (13, 22). Paeoniflorin, a monoterpene glucoside, is the principal bioactive constituent of peony root (40). Paeoniflorin has reportedly exhibited anti-inflammatory, immunoregulation, anti-arthritis, antihepatitis, pain-relieving, neuromuscular blocking, and antihyperglycemia effects (15, 32, 40, 41). Previous studies have shown that paeoniflorin suppresses TNF-α induced chemo-kine production in human dormal microvascular endothelial cells by blocking the NF-κB and ERK pathway (2). A recent investigation (article in Chinese) indicated that paeoniflorin effectively ameliorates the symptoms of oxazolone-induced colitis, but the underlying mechanism is not well established (44). On the basis of the key role TLR4 plays in intestinal inflammation, we investigated the effects of paeoniflorin on...
TLR4 expression and evaluated NF-κB and MAPK pathways in DSS-induced colitis.

MATERIALS AND METHODS

Animals. Healthy 8-wk-old female C57BL/6 mice (20 ± 2 g) were obtained from Shanghai Laboratory Animal Center, Shanghai, China and housed under a specific pathogen-free facility at room temperature (25°C) with alternating 12:12-h light-dark cycles. Standard laboratory chow and water were supplied ad libitum. This study was approved by the Animal Ethics Committee of Shanghai University of Traditional Chinese Medicine (SHUTCM). All mice were housed under a specific pathogen-free facility at SHUTCM and kept under the same laboratory conditions of temperature (25 ± 2°C) and lighting (12-h light-dark cycle) and were given free access to standard laboratory chow and tap water.

Induction of colitis and administration of paoniflorin. The experiment lasted for 10 days. Colitis was induced by giving 4% (wt/vol) DSS (molecular mass 36,000–50,000 Da, MP Biomedicals, Solon, OH) in drinking water for 7 days ad libitum. Eight-week-old female mice were randomly distributed into four groups (n = 10 per group): 

- **Group 1**, vehicle controls were administered 100 μl of 0.5% (wt/vol) methylcellulose by oral gavage once per day;
- **Group 2**, paoniflorin (dissolved in 0.5% methylcellulose) at a dose of 50 mg/kg of body wt via oral gavage once per day;
- **Group 3**, 100 μl of 0.5% (wt/vol) methylcellulose by oral gavage once per day and 4% (wt/vol) DSS in drinking water from day 3 to day 10;
- **Group 4**, paoniflorin by oral gavage (50 mg/kg) 3 days prior to or concurrently with DSS treatment and continued to the end of DSS treatment. Total gavage volume was identical for each group. At 4 h after receiving the last gavage, the mice were killed and the colon was removed. Paoniflorin (cat. no. 12-1001, HPLC ≥ 98%) was obtained from Shanghai R&D Center for Standardization of Traditional Chinese Medicine, Shanghai, China, and the dosage selection (50 mg/kg) was based on previous in vivo studies and was confirmed to result in effective alleviation of acute lung injury and arthritis in mouse disease models (40, 43).

Colitis evaluation. Mice were monitored daily for body weight, diarrhea, and bloody stool. Bloody diarrhea event was assessed as described before (3, 4). Following measurement of the colon length, the distal colon was taken and fixed in 10% (wt/vol) buffer formalin for H&E staining. Histological damage was assessed as a combined score of inflammatory cell infiltration (score 0–3) and mucosal damage (score 0–3) by a method previously described (5, 24, 36). For inflammatory cell infiltration in the colon mucosa, rare inflammatory cells (mononuclear infiltrates) in the lamina propria were counted as 0; increased numbers of inflammatory cells, including neutrophils in the lamina propria, were counted as 1; confluence of inflammatory cells, extending into the submucosa, were scored as 2; and a score of 3 was given for transmural extension of the inflammatory cell infiltration. For epithelial damage, absence of mucosal damage was counted as 0, discrete focal lymphoepithelial lesions were counted as 1, mucosal erosion/ulceration was counted as 2, and a score of 3 was given for extensive mucosal damage and extension through deeper structures of the bowel wall. The two subscores were added and the combined histological score ranged from 0 (no changes) to 6 (extensive cell infiltration and tissue damage).

Immunoblotting analysis. The mouse macrophage RAW264.7 cells were procured and cultured according to the guidelines of American Type Culture Collection (ATCC, Rockville, MD). When cells reached 70% confluence, paoniflorin (50 μM) was added and incubated for 2 h. Cells were overnight stimulated with LPS (2 μg/ml, Escherichia coli 055:B5, L6529—Ing, Sigma-Aldrich, St. Louis, MO) and then subjected to cell lysis. Colon tissues were disrupted by homogenization on ice and centrifuged at 4°C (12,000 g, 15 min) and the supernatants were collected. Equal amounts of protein (40 μg/lane) were separated on 10% SDS-PAGE and transferred to nitrocellulose membrane. Membranes were blocked in 5% (wt/vol) skim milk and incubated with antibodies against mouse TLR4(sc-293072, 1:1,000, Santa Cruz Biotechnology, CA). Phospho-p65 (sc-3033, 1:1,000, Cell Signaling Technology, Beverly, MA), IκBα (no. 4814, 1:1,000, Cell Signaling), phospho-ERK1/2 (no. 4377, 1:1,000, Cell Signaling), ERK1/2 (no. 4348, 1:1,000), phospho-JNK (no. 9255, 1:2,000, Cell Signaling), JNK (no. 9252, 1:1,000, Cell Signaling), phospho-p38 (no. 9215, 1:1,000, Cell Signaling), p38 (no. 9212, 1:1,000, Cell Signaling), and β-actin (no. 4970, 1:2,000, Cell Signaling). Blots were then washed three times with PBS containing 0.1% (wt/vol) Tween-20 (PBST) and incubated with horseradish peroxidase-conjugated secondary antibodies (Santa Cruz). Blots were again washed with PBST and then developed by enhanced chemiluminescence detection reagents (GE Healthcare, Pittsburgh, PA). The protein bands were quantified by the average ratios of integral optic density following normalization to the housekeeping gene β-actin expression.

RNA analysis. When RAW264.7 cells reached 70% confluence, cells were treated with or without paoniflorin (50 μM) for 2 h prior to LPS (2 μg/ml, Sigma-Aldrich) treatment for an additional 12 h. RNA was extracted from cultured cells or colon tissues by use of TRizol reagent (Life Technologies, Carlsbad, CA). Quantitative real-time PCR (qPCR) was performed by using cDNA generated from 3 μg total RNA with the SuperScript II Reverse Transcriptase kit (Life Technologies). The primer sequences used in PCR amplification are as follows: 5′-CTGAAGCCTGTGGCTTACAT-3′/5′-ACTACGCTCTCGCAGGG-3′ for mTLR2, 5′-TTCAGACCCGCTTGGTATATC-3′/5′-CCCCATTCAGGTAGGTGT-3′ for mTLR4, 5′-GAATCAAGATCGCTTCTGT-3′/5′-ACCCAGGTCTTAAATCTCC-3′ for mTLR5, 5′-AGTTGACCTGAAACTGTA-3′/5′-TGAAGAGGAAATACATAAACA-3′ for mMCP-1, 5′-GAAGCTTTTGCTCTGCT-3′/5′-GCTCTTGCTAGTATGTGC-3′ for mIFN-γ, 5′-CTGGAGAAGTGGCAAGAGG-3′/5′-AGACAAAGAGCTGGTGTCG-3′ for mTNF-α, 5′-ACCAGGCCGCTCCTCCTTAC-3′/5′-CATTTCCAGGACATTCCCAGA-3′ for miR-6, 5′-TCAGACTACCTCAACCAGGTC-3′/5′-ATGGTTGGTTCAGTCGATCC-3′ for miR-17, 5′-CAGGCTCTCTCTGTGGAT-3′/5′-TTGGCATAGCTC-3′ for miR-122, and 5′-CAGGCTCTCTCTGTGGAT-3′/5′-TTGGCATAGCTC-3′ for miR-122. The level of each miRNA was evaluated using the comparative ΔΔCt method and the values were normalized to endogenous reference β-actin.

Determination of TNF-α and IL-6 levels. Colon segments were homogenized in ice-cold PBS. The homogenates were centrifuged at 3,000 g for 10 min and the supernatants were assayed to determine levels of TNF-α and IL-6. RAW264.7 macrophages were pretreated with paoniflorin (50 μM) for 2 h and incubated with LPS (2 μg/ml, Sigma-Aldrich) for 12 h, the supernatants were harvested and the level of IL-6 was determined. The level of each cytokine was evaluated using ELISA kits according to the manufacturer’s protocols (R&D Systems, Minneapolis, MN) and the results were expressed in picograms per milligram of protein for tissue samples and picograms per milliliter for cell mediums.

Determination of MPO activity. 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 (5) and the values were expressed as units per milligram of protein.

NF-κB nuclear translocation and immunofluorescence. RAW264.7 cells were seeded in eight-chamber slides (BD Biosciences, Bedford, MA) at a density of 5 × 10^4 cells per well. Cells were allowed to adhere at 37°C overnight and paoniflorin (50 μM) was added for 2 h. Then cells were overnight stimulated with LPS (2 μg/ml, Sigma-Aldrich) and fixed with 4% (wt/vol) paraformaldehyde solution at 20°C for 10 min. After washing in PBS, cells were permeabilized with 0.3% (wt/vol) Triton X-100 in PBS at room temperature for 20 min.

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After incubation in blocking buffer of 0.1% (wt/vol) Triton X-100, 1% (wt/vol) BSA, and 3% (wt/vol) donkey serum, cells were then incubated with rabbit NF-κB p65 antibody (no. 8242, 1:50, Cell Signaling) overnight at 4°C. After washing in PBS, cells were further incubated with Alexa Fluor 488-conjugated donkey anti-rabbit IgG (A-21206, 1:500, Life Technologies) at room temperature for 45 min. To stain the nuclei, we added 1 μg/ml of DAPI (Life Technologies) at room temperature. Fluorescence photographs were obtained by use of an Olympus CKX41 fluorescence microscope.

NF-κB luciferase reporter assay. Human colorectal carcinoma HT-29 cells were purchased from ATCC (Manassas, VA), and 1×10⁶ HT-29 cells were electroporated with pGL4.32[luc2P/NF-κB RE/Hygro] reporter vector (Promega, WI) by use of the Lonza Nucleofector II instrument (Amaxa Biosystems, Germany) according to the manufacturer’s protocol. The pGL4.32 reporter is a NF-κB reporter vector containing NF-κB response elements and firefly luciferase gene. Cells were then seeded into 48-well plate following transfection.

After overnight incubation, cells were incubated with or without paeoniflorin (25, 50, and 100 μM) for 2 h followed by an additional incubation with LPS (2 μg/ml, Sigma-Aldrich) for 12 h. The body weight loss, diarrhea event rate, colon shortening, and colon histology. By contrast, paeoniflorin administration to DSS-exposed mice resulted in significant protection of the colon crypt structures and less severe histological inflammation (Fig. 1, E and F). This result strongly suggested that the inhibition of inflammatory infiltration was a mechanism for the protective effects of paeoniflorin in DSS-induced colitis. Next, to determine whether paeoniflorin could also ameliorate the severity on DSS-induced colitis, coadministration experiments were performed. Administering paeoniflorin at the same time as DSS treatment would replicate the clinical ease of administration that would occur in conjunction with inflammatory relapses in IBD. The results for the body weight loss, diarrhea event rate, colon shortening, and colon histology (Fig. 1, A–F) indicated that paeoniflorin coadministration with DSS can also decrease the severity of DSS-induced colitis even though the attenuated effect is less pronounced than using the preadministration schedule.

Paeoniflorin decreases TLR4 mRNA and protein expression. Considering TLR4 is an upstream signaling protein involved in NF-κB activation and the pathogenesis of IBD, we hypothesized that the protective effect of paeoniflorin is mediated by interference with TLR4. To determine the effect of paeoniflorin on TLR4 expression, qPCR and immunoblotting analyses were carried out. The results demonstrated that mRNA (Fig. 2A) and protein (Fig. 2B) expression of TLR4 were significantly induced in colon mucosa after 7-day DSS treatment compared with normal control group. The relative increase in mRNA and protein expression of TLR4 after DSS treatment was significantly downregulated in mice pretreated with paeoniflorin. The in vitro data performed in mouse macrophage RAW264.7 cells confirmed the results with (Fig. 2, C and D). The results indicated that paeoniflorin might decrease the severity of DSS-induced colitis via targeting TLR4 pathway. Indeed, to determine the effects of paeoniflorin treatment on alternative TLRs known to be dysregulated in IBD, namely, TLR2 and TLR5 mRNA expression abundance (6, 30, 37), qPCR was performed. Interestingly, TLR2 mRNA expression was significantly upregulated, whereas TLR5 mRNA expression remained unchanged in DSS-induced colitis. In addition, neither control nor inflamed colons exhibit significant difference in the expression of TLR2 and TLR5 after paeoniflorin preadministration (Fig. 2E). Collectively, the data strongly indicated that TLR4 but not TLR2 or TLR5 appears to account for the anti-inflammatory effect of paeoniflorin.

Paeoniflorin treatment blocks NF-κB activation in vivo and in vitro. NF-κB is a central regulator of immune response and NF-κB activation is indicated to be an important step in the development of human IBD (27). On the basis of this rationale, we hypothesized that the anti-inflammatory effect of paeoni-
Fig. 1. Preadministration (pre-) and coadministration (co-) of paeoniflorin (Paeo) attenuated clinical symptoms in dextran sulfate sodium (DSS)-induced colitis mice. A: body weight changes. Data plotted as percentage of basal body weight. B: occurrence of bloody diarrhea. Data plotted as percentage of total mice that had bloody diarrhea on different days (point of time) of DSS treatment. C: macroscopic view of the colon. D: colon length. E: representative H&E-stained colon sections (magnification ×200). F: histology score. Values were expressed as means ± SD (n = 10). *P < 0.05, **P < 0.01, ***P < 0.001 vs. DSS-treated group.
florin in response to DSS-induced colitis, correlated with the blockade of NF-κB activation. A significant increase ($P < 0.001$) in the protein expression of phospho-p65 was observed in colon mucosa of DSS-induced model group (Fig. 3A). Furthermore, DSS-induced IκBα phosphorylation/degradation and phosphorylation of NF-κB p65 were dramatically inhibited by coadministration DSS plus paeoniflorin ($P < 0.001$). In accordance with the in vivo data, paeoniflorin inhibited NF-κB-mediated luciferase activity in human colorectal carcinoma HT-29 cells by LPS treatment (25, 50, and 100 μM) in a dose-dependent manner (Fig. 3C). The results showed that mRNA expression of MCP-1, Cox2, IFN-γ, TNF-α, IL-6, and IL-17 was remarkably induced in inflamed colons of mice exposed to DSS (group 3). In contrast, the increase in inflammatory mediators and cytokines following DSS treatment was significantly decreased ($P < 0.001$) in mice receiving paeoniflorin administration (Fig. 4, A–F). The results indicated
that paeoniflorin ameliorates DSS-induced colitis which correlates with the suppression of NF-κB signaling.

Paeoniflorin reduces the production of TNF-α and IL-6. After 7 days of DSS administration, the levels of colonic TNF-α and IL-6 increased markedly (Table 1). Paeoniflorin administration significantly prevented the production of TNF-α and IL-6 in the inflamed colon. Consistent with the in vivo results, IL-6 secretion from RAW264.7 macrophages was enhanced by LPS treatment, but the enhancement was significantly suppressed by paeoniflorin administration (Fig. 4G). The data indicated that paeoniflorin inhibits the production of proinflammatory cytokines and thereby suppresses inflammatory responses.

Paeoniflorin decreases the activity of MPO in the inflamed colon. MPO activity, a marker for neutrophil infiltration into the inflamed tissue, was low in the colonic tissues of control mice (group 1 and group 2) and markedly increased in mice with DSS-induced colitis (Table 1). These results, along with the histological confirmation, demonstrate increased neutrophil infiltration in DSS-induced colitis mice. The increased MPO activity in mice with DSS-induced colitis was significantly reduced after administration of paeoniflorin. This finding suggested that paeoniflorin exerts anti-inflammatory effect by reducing neutrophil infiltration in the colonic mucosa.

LPS-mediated cytokine expression is blunted by paeoniflorin and directly dependent on TLR4. We have demonstrated that paeoniflorin decreases the expression of TNF-α and IL-6 (Fig. 4, D and E). To explore the mechanistic involvement of TLR4 in the regulation of cytokines expression by paeoniflorin, TLR4 gene expression was silenced by TLR4 siRNA transfection. As illustrated in Fig. 5A (top), treatment of RAW264.7 cells with TLR4 siRNA significantly decreased the expression of TLR4. Exposure of the control siRNA transfected RAW264.7 cells to
LPS caused a significant increase in the mRNA expression of TNF-α and IL-6 (data not shown), and these effects were prevented by that cotreatment with paeoniflorin (Fig. 5A, bottom). Interestingly, the regulatory effect of paeoniflorin was lost in cells lacking the expression of TLR4, indicating a key role for TLR4 in mediating the paeoniflorin effect (Fig. 5B). To complement the study described above, we overexpressed TLR4 in colon cancer HT-29 cells. A robust increase in the mRNA expressions of TNF-α and IL-6 was observed in LPS-stimulated HT-29 cells transfected with TLR4 expression vector pcDNA3-TLR4-YFP; this effect was attenuated by cotreatment with paeoniflorin (Fig. 5B).

Taken together, these results indicated that the effect of paeoniflorin in the downregulation of cytokines is dependent on TLR4 expression.

Paeoniflorin suppresses the MAPK pathway signaling. To investigate whether MAPK implicates in the regulatory effects of paeoniflorin on DSS-induced inflammation, we assessed the activation of signaling molecules including ERK1/2, JNK, and p38 MAPKs. As shown in Fig. 5C, DSS induced strong phosphorylation (activation) of ERK1/2, JNK, and p38 in the inflamed colon. Paeoniflorin treatment markedly inhibited the DSS-induced increase of phosphorylation of ERK1/2, JNK, and p38. The results suggested that MAPK signaling suppres-
sion may also contribute to the anti-inflammatory effect of paeoniflorin.

**DISCUSSION**

IBD, consisting of Crohn’s disease (CD) and ulcerative colitis (UC), is a chronic inflammatory disease of the gastrointestinal tract. The combined prevalence of CD and UC is ~300–400 cases/100,000 individuals in Western countries (17). Therapeutic strategies currently available for the management of IBD generally include administration of 5-aminosalicylates or sulfasalazine, antibiotics, glucocorticoids, immunosuppressive agents (6-mercaptopurine, azathioprine, methotrexate, cyclosporine, tacrolimus) and biologics such as anti-TNF agents (infliximab, adalimumab, certolizumab). However, despite their efficacy, some patients are unresponsive to these therapies and often suffer from numerous side effects that preclude the continuation of the treatment (17). Consequently, alternative medicine is becoming an increasingly attractive approach for the treatment of various IBD among patients unresponsive to or unwilling to take standard medicines (25). Among these alternative approaches is the use of herbal medicines; a substantial body of evidence demonstrates that various herbal ingredients or naturally occurring compounds present significant anticolitic effects (20, 39). However, limited scientific evidence regarding the mechanistic understanding of their actions has prevented their prevalent use (20).

**Table 1. Effects of paeoniflorin on MPO activity and the levels of TNF-α and IL-6 in DSS-induced colitis mice**

<table>
<thead>
<tr>
<th>Group</th>
<th>TNF-α, pg/mg pr</th>
<th>IL-6, pg/mg pr</th>
<th>MPO, U/mg pr</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>20.8 ± 1.9</td>
<td>14.8 ± 3.7</td>
<td>4.3 ± 0.5</td>
</tr>
<tr>
<td>Paeoniflorin</td>
<td>29.9 ± 2.8</td>
<td>15.5 ± 1.1</td>
<td>2.7 ± 0.3</td>
</tr>
<tr>
<td>DSS</td>
<td>212.7 ± 9.2‡</td>
<td>103.6 ± 6.9‡</td>
<td>19.6 ± 1.4‡</td>
</tr>
<tr>
<td>DSS + paeoniflorin</td>
<td>53.8 ± 3.4‡</td>
<td>42.3 ± 3.7†</td>
<td>13.7 ± 1.6†</td>
</tr>
</tbody>
</table>

Colon segments from mice (n = 6 per group) were excised and homogenized. The supernatants were assayed for the determination of the activity of MPO and the levels of TNF-α and IL-6. Values are expressed as means ± SD of triplicates of 2 independent experiments. *P < 0.001 vs. vehicle-treated group, †P < 0.01, ‡P < 0.001 vs. dextran sulfate sodium (DSS)-treated group.

**Fig. 5. Role of TLR4 in paeoniflorin-mediated downregulation of cytokines and effect of paeoniflorin on the suppression of MAPK pathway.**

**A:** RAW264.7 cells were electroporated with TLR4 siRNA (m) or control siRNA. Cells were treated with or without paeoniflorin (50 μM) and were stimulated with LPS (2 μg/ml) for 12 h. Cells were harvested and analyzed by Western blot (top) or qRT-PCR (bottom). **P < 0.01 vs. vehicle-treated control siRNA-transfected cells.**

**B:** HT-29 cells were electroporated with human TLR4 expression vector. Cells were treated as described above and analyzed by Western blot (top) or qRT-PCR (bottom). ***P < 0.001 vs. LPS-treated untransfected cells.***

**C:** expression of the phospho-ERK, phospho-JNK, and phospho-p-38 in colon tissues was analyzed by Western blot (n = 4). Quantification of the protein expression was performed by densitometric analysis of the blots. **P < 0.01, ***P < 0.001 vs. DSS-treated group.
Oral administration of DSS in mice induces colitis resembling human UC. This model corresponds well to the clinical signs of UC in human and can serve as a reliable model for the studies of this disease (26). In the present study, we demonstrated that pre- and coadministration of paeoniflorin significantly ameliorated the severity of DSS-induced colitis in mice. We have reported that baicalein, a natural flavonoid with anti-inflammatory activity, ameliorated DSS-induced colitis via targeting Cdx2/PXR axis and inhibiting NF-κB signaling genes (3). Since NF-κB activation is an important step in the progress of human IBD, we hypothesized that the antiinflammatory effect of paeoniflorin might be related to NF-κB inhibition. The results confirmed that paeoniflorin ameliorates DSS induced colitis by inhibiting NF-κB activation. Consistent with the in vivo inhibition of iNOS degradation and phosphorylation of NF-κB p65, in vitro evaluation of NF-κB activity suggested a direct role of paeoniflorin in the inhibition of NF-κB signaling: LPS treatment of mouse macrophage RAW264.7 cells resulted in nuclear translocation of NF-κB and could be reversed by paeoniflorin treatment; LPS treatment of transiently transfected human colon cancer HT-29 cells revealed paeoniflorin inhibited NF-κB luciferase reporter activity in a dose-dependent manner. Actually, we found that the administration of paeoniflorin not only inhibited NF-κB activity but also decreased the activity of MPO, reduced the production of inflammatory cytokines (TNF-α and IL-6), downregulated inflammatory mediators (MCP-1, Cox2, IFN-γ, TNF-α, IL-6, and IL-17), and limited the inflammatory (histological) response. Together, these molecular changes resulted in significant amelioration of DSS-induced colitis.

Although the pathogenesis of IBD is not fully understood, it is generally accepted that the inflammation is a result of an aberrant immune response to antigens of the host gut microbiota in genetically susceptible individuals (12). The intestinal lumen contains a vast array of different substances that may interact with the host, such as dietary factors, microbial components, and environmental pollutants (19). Many of these xenobiotics interact with NF-κB via activation of Toll-like receptors such as TLR4 (27). Recently, TLR4-mediated NF-κB activation in the intestine was proved to play a causative role in the disruption of mucosal homeostasis (7). TLR4 activation in fact leads to intestinal injury and generally contributes to the pathogenesis of IBD (10). TLR4 knockout mice have long been used for the study of TLR4 signaling in the pathogenesis of IBD; however, the underlying effects are still elusive. It has been reported that TLR4 knockout mice exposed to DSS developed significantly less inflammation and deletion of TLR4 attenuated the colitis in TLR5 knockout mice (11, 35). Recent work by Fukata et al. (10) demonstrated that the absence of TLR4 lowered the susceptibility to colitis-associated colorectal cancer. In contrast, lacking TLR4 expression seems to be also detrimental. TLR4-deficient mice were reported to have worse colitis after DSS than wild-type counterparts but intriguingly were protected from colitis-associated cancer (29, 42). The molecular basis for this discrepancy is unclear, whereas some complementary mechanisms might be activated in the absence of TLR4. In our study, TLR4 was upregulated in the inflamed colons as well as in LPS-stimulated RAW264.7 cells; however, the upregulation of TLR4 was significantly reversed by paeoniflorin treatment. To explore the mechanistic involvement of TLR4 in the action of paeoniflorin, colon cancer HT-29 cells were transfected with human TLR4 expression vector. We found that overexpression of TLR4 in HT-29 cells potentiated LPS-induced upregulation of TNF-α and IL-6, whereas this effect was attenuated by cotreatment with paeoniflorin. In addition, we observed that exposure to the TLR4 ligand LPS caused a significant increase in the mRNA expression of TNF-α and IL-6 in RAW264.7 cells transfected with the control siRNA, and these effects were effectively prevented by that cotreatment with paeoniflorin, whereas silencing the expression of TLR4 almost completely abrogated the ability of paeoniflorin to counteract the effects of LPS. Thus our results support the hypothesis that paeoniflorin appears to decrease the proinflammatory cytokines through a TLR4-dependent manner.

Several previous studies have demonstrated that paeoniflorin regulated the key molecules involved in inflammation. For example, paeoniflorin suppresses the inflammatory mediators IL-1, TNF-α, and PGE2 in type II collagen induced arthritic rats (40). Zhou et al. (43) have showed that paeoniflorin protects against LPS-induced acute lung injury through downregulation of the activation of p38, JNK, and NF-κB pathways. Chen et al. (2) showed that paeoniflorin suppresses TNF-α induced chemokine production in human dermal microvascular endothelial cells, which is relevant to NF-κB and ERK pathways suppression. Wu et al. (38) suggested that paeoniflorin suppresses NF-κB activation through modulation of iNOS and enhancement of 5-fluorouracil-induced apoptosis in human gastric carcinoma cells. Here we reported that paeoniflorin treatment also significantly inhibited the DSS-induced activation of ERK1/2, JNK, and p38 MAPKs. Taken together, these in vitro and in vivo results provide the first evidence that the beneficial effects of paeoniflorin in DSS-induced colitis might be related to the downregulation of TLR4 expression and the blockade of the activation of NF-κB and MAPK pathways. These results may give insight into the further evaluation of paeoniflorin as a therapeutic agent or potential adjunct to IBD therapy.

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