Pien Tze Huang ameliorates DSS-induced colonic inflammation in a mouse colitis model through inhibition of the IL-6/STAT3 pathway

LI LI1,2, ALING SHEN1,3, JIANFENG CHU1,3, THOMAS J. SFERRA4, SENTHILKUMAR SANKARARAMAN3, XIAO KE5, YOUQIN CHEN4 and JUN PENG1,3

1Academy of Integrative Medicine, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122; 2Department of Disease Prevention and Healthcare, Fujian Provincial Hospital, Fuzhou, Fujian 350001; 3Fujian Key Laboratory of Integrative Medicine on Geriatric, Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350122, P.R. China; 4Department of Gastroenterology, Rainbow Babies and Children’s Hospital, Case Western Reserve University School of Medicine, Cleveland, OH 44106, USA; 5Department of Gastroenterology, Second Affiliated Hospital of Fujian University of Traditional Chinese Medicine, Fuzhou, Fujian 350003, P.R. China

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Abstract. Interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) pathway plays essential roles in the development of inflammatory diseases including ulcerative colitis (UC). Therefore, suppression of IL-6/STAT3 signaling provides a promising therapeutic strategy in UC. Pien Tze Huang (PZH), a well-known traditional Chinese formula, has been used in China and Southeast Asia for centuries as a folk remedy for various inflammatory diseases. However, the molecular mechanisms of its anti-inflammatory effects remain to be elucidated. In the present study, we generated a mouse colitis model by using dextran sulfate sodium (DSS) and evaluated the therapeutic efficacy of PZH against UC by observing the clinical manifestations. We found that PZH obviously alleviated DSS-induced colitis symptoms, including body weight loss, rectal bleeding and stool consistency. In addition, administration of PZH profoundly prevented DSS-induced colon shortening, and ameliorated colonic histopathological changes such as mucosal ulceration, infiltration of inflammatory cells, crypt distortion and hyperplastic epithelium. Moreover, PZH markedly inhibited the serum level of the inflammatory biomarker serum amylose A (SAA) in UC mice. Furthermore, PZH treatment significantly inhibited DSS-induced expression of IL-6 in colon tissues. Finally, the increased phosphorylation level of STAT3, induced either by DSS in experimental mice or by IL-6 in the differentiated human colorectal carcinoma cells, was significantly suppressed by PZH. These results suggest that the inhibition of IL-6/STAT3 signaling is a potential mechanism by which PZH is used in the treatment of UC.

Introduction

Inflammatory bowel disease (IBD), typically referring to ulcerative colitis (UC) and Crohn's disease (CD), is a group of chronically inflammatory disorders of gastrointestinal tract without specific etiology (1-4). Although these inflammatory conditions have been treated with 5-aminosalicylic acid derivatives, corticosteroids and immunosuppressants (5), most of these currently used treatments are inadequate due to severe side effects such as systemic immunosuppression (6-8). Thus, there is a need to develop novel and safer therapeutic agents. Recently, natural product-based treatments, particularly Traditional Chinese Medicine (TCM), have received great interest as they have relatively few side effects and have been used as alternative remedies for a variety of diseases including IBD (9-12).

The pathogenic mechanisms underlying IBD development are complex and heterogeneous, with the involvement of multiple cellular transduction pathways including interleukin-6 (IL-6)/signal transducer and activator of transcription 3 (STAT3) signaling. IL-6 is an important pro-inflammatory cytokine that has been shown to play a potential role in the pathogenesis of IBD. Elevated IL-6 levels have been detected in serum, inflammed colonic mucosal tissues and cultivated lamina propria mononuclear cells in IBD patients (13-16). Moreover, IL-6 level is correlated with the inflammatory...
Materials and methods

**Materials and reagents.** Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), penicillin-streptomycin, Trypsin-EDTA were obtained from Life Technologies Corporation (Grand Island, NY, USA). A 24-well cell culture insert was purchased from BD Biosciences (Franklin Lakes, NJ, USA). DSS (molecular weight, 40,000 kDa) was purchased from Sigma Chemicals (Portland, OR, USA), respectively. All the other chemicals, unless otherwise stated, were obtained from Sigma Chemicals (St. Louis, MO, USA).

**Preparation of PZH.** PZH was obtained from and authenticated by the sole manufacturer Zhangzhou Pien Tze Huang Pharmaceutical Co., Ltd. (Zhangzhou, China; Chinese FDA approval no. Z35020242). The stock solution of PZH was prepared by dissolving PZH powder in saline to a concentration of 20 mg/ml, followed by sonication for 30 min. The working concentrations for cell-based experiments were made by diluting the stock solution in the cell culture medium.

**Animals.** Seven-week-old male BALB/c mice (weight, 20-22 g) were purchased from Shanghai SLAC Laboratory Animal Co., Ltd. (Shanghai, China) and were acclimatized for 1 week before the experiment. Animals were housed individually in a room maintained at 22°C under a 12-h day/night cycle. Food and water were given *ad libitum* throughout the experiments. The animal experiments conducted in this study were approved by the Animal Care and Use Committee of Fujian University of Traditional Chinese Medicine.

**In vivo mouse colitis study.** Mouse colitis model was established as we described previously (51). Briefly, acute colitis was induced by administration with 3% DSS in the drinking water for 8 days. On the first day of model construction, the animals were randomly divided into three groups (n=5): The normal control group in which mice received neither DSS stimulation nor PZH treatment; the DSS-induced model or PZH-treated group in which mice received DSS stimulation and were given intra-gastric administration with saline or 234 mg/kg, respectively, daily for 12 days. The progression of colitis was monitored in a blinded manner, including measurement of body weight, evaluation of stool consistency, and presence of rectal bleeding tested by guaiac paper. Disease activity index (DAI) was represented as the sum of scores for weight loss, stool consistency and rectal bleeding (Table I).

**Sample preparation.** At the end of the experiment, the animals were anaesthetized with Avertin. Blood was collected via right heart ventricle puncture in lightly heparinized syringes and kept on ice. Sera were separated by 5 min centrifugation at 5,000 x g and stored at -80°C prior to the analysis.

Table I. Score of the disease activity index.

<table>
<thead>
<tr>
<th>Score</th>
<th>Weight loss</th>
<th>Rectal bleeding</th>
<th>Stool consistency</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>0%</td>
<td>Normal</td>
<td>Normal</td>
</tr>
<tr>
<td>1</td>
<td>1-5%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>2</td>
<td>6-10%</td>
<td>Positive hemoccult</td>
<td>Loose</td>
</tr>
<tr>
<td>3</td>
<td>11-20%</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>4</td>
<td>&gt;21%</td>
<td>Gross bleeding</td>
<td>Diarrhea</td>
</tr>
</tbody>
</table>

Disease activity index was calculated as the sum of scores of each category.
protocol. Protein concentrations were determined by BCA protein assay kit.

Histopathological evaluation. The formalin fixed section of distal colons were processed and stained with hematoxylin and eosin (H&E) and evaluated under light microscopy in a blinded manner by an experienced pathologist.

Measurement of serum SAA level or expression of IL-6 in colon by ELISA. The level of SAA in the sera and the expression of IL-6 in colonic tissues were measured using ELISA kits according to the manufacturer's protocol. Absorbance was read at 450 nm using a microplate reader (model ELX800; BioTek Instruments, Inc., Winooski, VT, USA). All the samples were analyzed in triplicate.

Western blot analysis. Equivalent amounts of protein were resolved in 12% Novex Bis-Tris gel electrophoresis (NuPAGE; Life Technologies Corporation). Proteins were then transferred into nitrocellulose membranes in an iBlot Western Blotting system (Invitrogen, Grand Island, NY, USA). The membranes were blocked for 1 h at room temperature with super SuperBlock® Blocking Buffers (Pierce Biotechnology, Inc.). They were then incubated at 4˚C overnight with primary antibodies against p-STAT3 (rabbit, polyclonal, 1:1,000, CST, cat. no. 9131), regular STAT3 (rabbit, polyclonal, 1:1,000, CST, cat. no. 9132) and β-actin (rabbit, polyclonal, 1:2,000, CST, cat. no. 4967) in blocking buffer. After the membranes were washed, they were further incubated with appropriate horse-radish anti-rabbit IgG, HRP-linked antibody (1:5,000, CST, cat. no. 7074) for 1 h at room temperature. The membranes were analyzed using enhanced chemiluminescence Plus reagents and scanned with the Storm Scanner (Amersham Pharmacia Biotech, Inc., Piscataway, NJ, USA).

Cell culture. Human colon cancer Caco-2 cells were purchased from the American Type Culture Collection (Rockville, MD, USA). Cells (passages 20-40) were grown in DMEM supplemented with 20% (v/v) FBS, 1,000 mg/l of glucose, 50 U/ml penicillin and 50 µg/ml streptomycin in a 37°C humidified incubator with 5% CO₂. Caco-2 cells usually reached confluence 3 days after seeding, and differentiated into enterocyte-like cells 18-20 days post-confluence. To test the effect of PZH on IL-6-induced phosphorylation of STAT3, we seeded these fully differentiated cells in 24-well biocameral inserts (area, 0.33 mm²; pore size, 0.4 µm insert). On the day of the experiment, the medium was removed and supplemented with 0.5% FBS medium. Differentiated Caco-2 cells (20 days post-confluence) in 24-well biocameral inserts were pre-incubated with 0.5 mg/ml of PZH for 2 h followed by stimulation with 10 ng/ml of IL-6 for 30 min.

Measurement of cell viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. Differentiated Caco-2 cells (20 days post-confluence) in 96-well plates were treated with various concentrations of PZH for 24 h. The cytotoxic effect of PZH on Caco-2 cells was examined by the MTT colorimetric assay. Briefly, MTT (100 µl) (0.5 mg/ml in PBS) was added to each well, and the samples were incubated for an additional 4 h at 37°C. The purple-blue MTT formazan precipitate was dissolved in 100 µl DMSO. The absorbance was measured at 570 nm using a spectrophotometer reader (Model ELX800; BioTek Instruments, Inc., Winooski, VT, USA).

Statistical analysis. Data were presented as mean ± SD for the indicated number of independently performed experiments. The data were analyzed using the SPSS package for Windows (version 17.0; SPSS, Inc., Chicago, IL, USA). Statistical analysis was carried out using Student's t-test and one-way ANOVA, followed by LSD's test or Dunnett's test. Differences with P<0.05 were considered to be statistically significant.

Results

**Result**

PZH potentially inhibits the development of DSS-induced UC in mice. To determine whether PZH could inhibit the development of UC, we evaluated the clinical manifestations in experimental mice. The DAI was calculated based on the observation of body weight, stool consistency and rectal bleeding.
As shown in Fig. 1, upon DSS stimulation mice exhibited apparent manifestations of colitis which was characterized by body weight loss, diarrhea and rectal bleeding. Administration of PZH significantly ameliorated DSS-induced colitis symptoms. The DAI score of normal control, DSS-stimulated model or PZH-treated group was 0.6±0.54, 10.4±1.67 or 4.0±0.71, respectively (P<0.05; Fig. 1A). Moreover, colons were harvested from mice in each group after sacrifice; and length from cecum to anus was measured. We found that PZH treatment profoundly prevented DSS-induced colon shortening. The average colonic length in normal control, model or PZH-treated group was 7.74±0.34, 3.6±0.41 or 6.06±0.38 cm, respectively (P<0.05; Fig. 1B). Taken together, PZH is potent in suppressing the development of UC in vivo.

**PZH alleviates colon histological damages in mice with DSS-induced UC.** We next evaluated the histopathological changes of colon tissues through H&E staining. As shown in Fig. 2, the normal control mice displayed normal colonic histology with an intact epithelium, well-defined gland lengths and no leukocyte infiltration in the mucosa, whereas DSS induced severe colonic histological damages such as mucosal ulceration, inflammatory cell infiltration, crypt distortion and hyperplastic epithelium. However, PZH treatment significantly alleviated DSS-induced histopathological changes in colonic mucosa.

**PZH reduces SAA level in mice with DSS-induced UC.** It has been shown that the level of the inflammatory marker SAA is increased in UC patients. We therefore performed ELISA assay to examine SAA level in experimental mice. As shown in Fig. 3, PZH treatment significantly inhibited DSS-induced increase of serum level of SAA in UC mice. The SAA level in mice of normal control, model or PZH-treated group was 304.9±62.8, 1,667.9±202.9 or 558.2±92.5 µg/ml, respectively (P<0.05).

**PZH suppresses IL-6/STAT3 signaling pathway both in mice with DSS-induced UC and in inflammatory intestinal epithelial cells.** To investigate the mechanism of the anti-inflammatory activity of PZH, we evaluated the activation of IL-6/STAT3 pathway in colon tissues of experimental mice. The protein expression of IL-6 in colon tissues was determined by ELISA assay and STAT3 activation was examined by western blot analysis using antibody that recognizes STAT3 phosphorylation at Tyr705. As shown in Fig. 4, the expression of IL-6 and the phosphorylation level of STAT3 in DSS-induced UC mice was significantly increased, as compared to that in normal control mice; which, however, was significantly inhibited by PZH treatment.

By stimulating the differentiated human colorectal carcinoma Caco-2 cells with IL-6, we generated an inflammatory cell model of human intestinal epithelium to examine the in vitro effect of PZH on IL-6/STAT3 pathway. As shown in Fig. 5, IL-6 stimulation resulted in a significant increase of pSTAT3 level in Caco-2 cells. However, PZH treatment profoundly inhibited IL6-induced STAT3 phosphorylation. The levels of non-phosphorylated STAT3 remained unchanged after the treatment with IL-6 and/or PZH.

**PZH did not display cytotoxicity in intestinal epithelial cells.** To exclude the possibility that the in vitro suppressive activity of PZH on STAT3 pathway was due to cytotoxicity, we determined its effect on the viability of differentiated Caco-2 cells.
using MTT assay. As shown in Fig. 6, Caco-2 cell viability was not affected by treatment with PZH, suggesting that the inhibitory effect of PZH on IL-6-induced STAT3 activation in intestinal epithelial cells did not result from its cytotoxic action.

Discussion

Despite recent advances in the drug treatment of IBD, many currently used pharmacotherapies contain intrinsic side effects such as systemic immunosuppression, which limits their long-term use (5-8). Natural products, including TCM, have received great interest since they have long been used clinically to treat inflammatory diseases and are relatively toxicity-free (9-12). PZH is a well-known TCM formula which has been used in China and Southeast Asia for centuries to clinically treat a variety of inflammatory diseases (33-50). However, the precise mechanism of anti-inflammatory activity of PZH remains largely unknown.

Using a DSS-induced mouse colitis model, in the present study, we evaluated the therapeutic efficacy of PZH against UC. We found that PZH significantly ameliorated DSS-induced colitis symptoms, including body weight, stool consistency and rectal bleeding. In addition, the administration of PZH profoundly prevented DSS-induced colon shortening, and alleviated colonic histopathological changes such as mucosal ulceration, infiltration of inflammatory cells, crypt distortion and hyperplastic epithelium. Moreover, PZH markedly inhibited the DSS-induced increase in serum levels of SAA, one of the inflammatory biomarkers which is commonly elevated in patients with UC. Thus, PZH is potent in suppressing the development of UC.

The IL-6/STAT3 pathway is an important signaling pathway that mediates inflammatory response. As a critical pro-inflammatory cytokine, IL-6 plays essential roles in the development of inflammatory diseases including IBD. Elevated IL-6 levels have been detected in the serum, inflammed colonic mucosal tissues and cultivated lamina propria mononuclear cells in IBD patients and in murine model with acute inflammation (13-16). In addition, serum IL-6 level is positively correlated with severity of intestinal histopathology (17-23). Generally, IL-6 exerts its bioactivities via binding to the sIL-6R, leading to the activation of JAKs and the downstream effectors such as STAT3 (24-27). Activated STAT3 proteins in the cytoplasm form homodimers and translocate to the nucleus to regulate the expression of various critical genes mediating antiapoptotic activities (28,29). Constitutive activation of STAT3 has been commonly found in lamina propria mononuclear cells from IBD patients, resulting in the resistance against apoptosis in...
these cells and eventually the progression of IBD (21,30,31). Thus, the IL-6/STAT3 pathway has become a major target in the treatment of IBD. Using ELISA assay we found that PZH treatment significantly inhibited the DSS-induced expression of IL-6 in UC mice. Moreover, the increased phosphorylation of STAT3, induced by DSS in experimental mice or by IL-6 in the differentiated human colorectal carcinoma cells, was profoundly suppressed by PZH.

In conclusion, in the present study we report, to the best of our knowledge, for the first time that PZH attenuates intestinal inflammation in murine colitis model probably through inhibition of the IL-6/STAT3 pathway, demonstrating its potential clinical value in the treatment of IBD.

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Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

Authors' contributions

JP and YC conceived and designed the experiments. LL, AS, JC and XK conducted the animal experiments. LL and TJS performed western blot and data analysis. AS and SS conducted ELISA and analysis. LL, JP and YC wrote and revised the manuscript.

Ethics approval and consent to participate

The animal experiments conducted in this study were approved by the Animal Care and Use Committee of Fujian University with protocol number 2018J01229.

Consent for publication

Not applicable.

Competing interests

The authors declare that they have no competing interests.

References


