Original Article
Astragaloside II alleviates the symptoms of experimental ulcerative colitis in vitro and in vivo

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Abstract: Background: Ulcerative colitis (UC) is a chronic inflammatory intestinal disease, and its morbidity is rising worldwide. Previous study indicated that astragaloside II (AS II), a monomeric compound, was used to treat bowel disease. However, the effects of AS II on UC remains unclear. Thus, this study aimed to investigate the therapeutic effects of AS II on experimental UC in vitro and in vivo. Methods: CCD-18Co cells were stimulated by 1 μg/mL LPS to mimic UC in vitro. In addition, dextran sulfate sodium (DSS)-induced UC mouse model was established in vivo. CCK-8 assay was used to detect cell proliferation in vitro. Moreover, the concentrations of inflammatory factors interleukin 6 (IL-6), tumor necrosis factor-α (TNF-α), interleukin 1β (IL-1β), nitric oxide (NO), superoxide dismutase (SOD) and malondialdehyde (MDA) in CCD-18Co cells and colon tissues were determined by ELISA, respectively. Meanwhile, the expressions of hypoxia-inducible factor 1α (HIF-α), phospho-inhibitor of NF-κB (p-IκB) and phospho-NF-κB p65 (p-p65) were detected by western blotting in vitro and in vivo, respectively. Results: In this study, the levels of pro-inflammatory cytokines TNF-α, IL-1β and IL-6 were significantly increased in lipopolysaccharide (LPS)-stimulated CCD-18Co cells. However, LPS-induced inflammatory response was markedly alleviated by AS II. In addition, LPS-induced HIF-α, p-IκB and p-p65 proteins increases were markedly ameliorated by AS II treatment. Moreover, AS II reduced disease activity index (DAI) scores and increased the colon lengths in DSS-treated mice. Meanwhile, AS II decreased the levels of IL-6, TNF-α, IL-1β, NO, MPO and MDA, and increased the level of SOD in colon of DSS-treated mice. Furthermore, AS II downregulated the expressions of HIF-α, p-IκB and p-p65 in DSS-induced UC in mice. Conclusion: Our findings indicated that AS II could alleviate inflammatory response in LPS-induced CCD-18Co cells and in DSS-induced UC in mice. In conclusion, AS II may serve as a potential agent for the treatment of UC.

Keywords: Astragaloside II, lipopolysaccharide, dextran sulfate sodium, ulcerative colitis

Introduction
Ulcerative colitis (UC) is a chronic and inflammatory disease of the rectum and colon [1]. Patients with UC in adolescence or young adulthood were diagnosed with symptoms of diarrhea, abdominal pain, and rectal bleeding [2]. The morbidity of UC was increasing with globalization [3]. 15% of the patients had been hospitalized during the exacerbations [4]. Conventional colonoscopy is an important detection method to evaluate the disease severity degree in patients with UC [5, 6]. However, conventional colonoscopy has some limitations, including adverse events and low patient compliance when patients receive treatment [7, 8]. Therefore, it is necessary to explore better therapeutic strategies for treating UC.

Astragaloside II (AS II) is a Chinese monomeric compound, which extracted from the Chinese traditional herb Astragalus membranaceus [9]. Previous reports demonstrated that AS II exhibited multiple pharmacological activities, such as anti-tumor activity, anti-bacteria activity, anti-inflammation activity and so on [10-12]. In addition, AS II could reduce cell proliferation and induce cell apoptosis in cancer cells [13]. Moreover, AS II could repair irritable bowel disease by activating the mTOR pathway [9].

It has been shown that inflammatory cell infiltration could lead to decreased mucosal perfu-
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...in inflamed mucosal tissues, and thus could activate hypoxia-inducible factor-1-alpha (HIF-α) [14]. HIF-α is transcription factor, which could link inflammatory pathways, including nuclear factor-kappa B (NF-κB) pathway [15]. NF-κB, a pro-inflammatory cytokine, which was associated to the development and pathogenesis of UC [16]. Previous study indicated that activation of NF-κB could increase the release of proinflammatory cytokines TNF-α, IL-1β and IL-6 in DSS-induced UC mice [17]. In addition, colon length shortening and colonic pathological damage were observed in a mouse model of DSS-induced UC [17]. However, the anti-inflammatory effect of ASII on DSS-induced experimental UC in mice remains unclear. Therefore, this study aimed to explore the effect of ASII on experimental UC in vitro and in vivo.

Materials and methods

Cell line and cell culture

Human colon fibroblast CCD-18Co was purchased from the American Type Culture Collection (ATCC, Rockville, MD, USA). Cells were incubated in Eagle’s Minimum Essential Medium (ATCC), supplemented with 10% fetal bovine serum (FBS, Gibco, Carlsbad, CA, USA), penicillin and streptomycin (100 U/ml) in a humidified 5% CO₂ incubator at 37°C.

Cell viability detection

Cell viability was detected using the Cell Counting Kit-8 (CCK-8, Beyotime Biotechnology, Beijing, China) according to the manufacturer’s protocols. CCD-18Co cells (5×10³ cells/well) were plated into a 96-well plate and cultured overnight. Then, AS II (0, 0.1, 0.33, 1, 3 μM) were added into CCD-18Co cells at 37°C for 48 h. After that, 10 μL of CCK-8 reagent was added into each well. The absorbance at a wavelength of 450 nm was measured using a microplate reader (Bio-Rad Laboratories, Benicia, California, USA). LPS was provided by sigma (Sigma, St. Louis, MO, USA). AS II was obtained from MCE (Med Chem Express, Monmouth Junction, NJ, USA).

Enzyme-linked immunosorbent assay (ELISA) for detection of pro-inflammatory factors

CCD-18Co cells were treated with LPS (1 μg/mL) for 0, 12, 24 and 48 h to produce inflammatory mediators. In addition, CCD-18Co cells were treated with 1 μM AS II and 1 μg/mL LPS for 48 h. Samples of the supernatant were obtained from CCD-18Co cells. ELISA was used to detect the levels of IL-6, TNF-α and IL-1β in cell culture supernatant. IL-6, TNF-α and IL-1β ELISA kits were obtained from Nanjing Jiancheng Bioengineering Institute (Nanjing, China).

qRT-PCR

The total RNA from CCD-18Co cells were extracted by TRizol reagent (Thermo Fisher Scientific, Waltham, MA, USA) according to the manufacturer’s instructions. After that, cDNA was synthesized using the cDNA Reverse Transcription Kit (Thermo Fisher Scientific). Later on, real-time PCR was performed using a SYBR premix Ex Taq II kit (TaKaRa, Dalian, China) on an ABI 7900HT instrument (ABI, NY, USA). HIF-α, F: 5’-CCAGTGGACTTGGGGCCTTG-3’; R: 5’-AAAGGCAAAGACACCCCGGA-3’. GAPDH, F: 5’-ATGGCCTTCCGTGTTCCTAC-3’; R: 5’-CTTT-AACAGTTGTCGTTGA-3’. Relative quantification of gene expression was performed using the 2^ΔΔCT as fold changes. The primers for p-p65 was obtained from GenePharma (Shanghai, China).

Western blotting

CCD-18Co cells and colonic samples were homogenized using RIPA buffer in ice-cold homogenization. Bradford Protein Assay Kit (Beyotime, Biotechnology) was used to assess the quantity of protein. Equal quantity of proteins (40 μg) in lysate were then separated on 10% SDS polyacrylamide gel electrophoresis. After that, the proteins were transferred onto a polyvinylidene fluoride membrane (PVDF, Thermo Fisher Scientific). The membranes were blocked with TBST containing 5% nonfat milk for 2 h, and then membranes were applied with primary antibodies overnight at 4°C as follows: anti-HIF-α (1:1000), anti-p-p65 (1:1000), anti-p65 (1:1000), anti-p-κB (1:1000), anti-κB (1:1000) and anti-β-actin (1:1000). Then, the PVDF membranes were incubated with secondary antibody anti-rabbit (1:5000). The protein was detected by enhanced chemiluminescence (ECL) system (Bio-Rad, Hercules, CA, USA). The density of blots for targets were normalized to β-actin. All antibodies were purchased from Abcam (Cambridge, UK).
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**DSS-induced ulcerative colitis in mice**

Male BABL/c mice (20-22 g) were provided by Kay Biological Technology (Shanghai, China). The animals were housed according to the guideline of National Institutes Health for the Care and Use of Laboratory Animals. 48 mice were randomly separated into 8 experimental groups (n = 6): group I, control; group II, AS II_10 mg/kg; group III, AS II_30 mg/kg; group IV, AS II_50 mg/kg; group V, AS II_80 mg/kg; group VI, model group (DSS); group VII, DSS + 30 mg/kg AS II; group VIII, DSS + 50 mg/kg AS II.

The mice were treated with AS II (dissolved in 2% Tween 20 in PBS) via oral gavage once per day for 10 days in the morning. On the day 3, UC were induced by 3% (w/v) DSS added to drinking water for 5 consecutive days. The body weights of mice were monitored every day. The DAI score and was determined as previously reported [18]. In the termination of the study, the mice were euthanized using CO₂, and the colon was removed from animals. The length of colon was measured. All experimental procedures were approved by the Ethical Committee of Nanjing University of Chinese Medicine. DSS was obtained from Sigma. National Institutes of Health guide for the care and use of laboratory animals was followed.

**Histological evaluation**

The colonic samples were fixed in 10% formalin, and then embedded in paraffin for histological evaluation. The colonic specimens (5 μm) were stained with hematoxylin & eosin (H&E), and then observed by a light microscope. The grading of histological damage was assessed as described previously [19].

**Cytokine analysis by ELISA**

The colonic samples were weighed, and homogenized for 3 min on ice using a homogenizer. The levels of IL-6, TNF-α, IL-1β and NO in the colon homogenates were measured with ELISA kits (Nanjing Jiancheng Co., Nanjing, China) in accordance with the manufacturer’s instructions.

**Measurement of MPO, MDA and SOD**

The distribution number of neutrophils in colonic samples was detected by (myeloperoxidase) MPO activity. Colonic samples were weighed, and homogenized using reaction buffer. Then, MPO activity assay kit (Nanjing Jiancheng Co., China) was applied to assess the MPO activity. The activity of SOD and the content of MDA were determined using SOD or MDA activity assay kits according to manufacturer’s protocols (Nanjing Jiancheng Co., China).

**Immunohistochemistry (IHC) analysis**

p-p65 was detected by IHC staining according to the methods reported before. The samples were incubated with the primary antibodies overnight at 4°C. Later on, biotinylated immunoglobulin cocktail of goat anti-rabbit IgG was applied at room temperature. The colon specimens were observed using a fluorescence microscope.

**Statistical analysis**

Each group were performed at least three independent experiments and all data were presented as the mean ± standard deviation (SD). Statistical analysis was analyzed by GraphPad Prism v7.0 (GraphPad Software, Inc., La Jolla, CA, USA). Protein expressions of HIF-α, p-p65 and p-IκB and the levels of proinflammatory cytokines IL-6, TNF-α, IL-1β were analyzed with one-way analysis of variance (ANOVA) followed by Tukey’s test. In addition, histological evaluation and IHC assays were analyzed with one-way analysis of variance (ANOVA) followed by Tukey’s test. P<0.05 was considered statistically significant.

**Results**

AS II attenuated oxidative stress damage in LPS-stimulated CCD-18Co cells via decreasing the production of inflammatory factors

The chemical structure of AS II was indicated in Figure 1A. CCK-8 assay was used to assess the potential cytotoxic effect of AS II on CCD-18Co cells. As indicated in Figure 1B, 3 μM AS II significantly decreased the viability of CCD-18Co cells, compared with control group. Meanwhile, 1 μM AS II had very limited on the cytotoxicity of CCD-18Co cells. Therefore, 1 μM AS II was utilized in following in vitro experiments. As shown in Figure 1C-E, LPS significantly increased the levels of IL-6, TNF-α and IL-1β in CCD-18Co cells, which were obviously reversed by AS II. In addition, the results of NO, SOD and MDA activity assays indicated that LPS signifi-
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Figure 1. AS II attenuated oxidative stress damage in LPS-stimulated CCD-18Co cells via decreasing the production of inflammatory factors. A. The chemical structure of AS II. B. CCD-18Co cells were treated with AS II (0, 0.1, 0.33, 1, 3 μM) for 48 h. CCK-8 assay was used to detect the viability of CCD-18Co cells. C. CCD-18Co cells were treated with LPS (1 μg/mL) for 0, 12, 24 and 48 h. In addition, CCD-18Co cells were treated with 1 μM AS II and 1 μg/mL LPS for 48 h. The level of IL-6 in the culture media was measured with ELISA. D. The level of TNF-α in the culture media was measured with ELISA. E. The level of IL-β in the culture media was measured with ELISA. F. CCD-18Co cells were treated with 1 μg/mL LPS or 1 μM AS II for 48 h. The level of NO in cells was measured with ELISA. G. The level of SOD in cells was measured with ELISA. H. The level of MDA in cells was measured with ELISA. *P<0.05, **P<0.01 compared with 0 h group; ***P<0.01 compared with 48 h group.
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Significantly decreased the level of SOD, and increased the levels of NO and MDA in CCD-18Co cells. However, the effect of LPS on the productions of NO, SOD and MDA were obviously reversed in the presence of AS II (Figure 1F-H). These data indicated that AS II could decrease the production of inflammatory factors and attenuate oxidative stress damage in LPS-stimulated CCD-18Co cells.

**AS II inhibited the levels of HIF-α, p-p65 and p-IκB in LPS-treated CCD-18Co cells in vitro**

We next investigated whether AS II exhibited anti-inflammation effect in LPS-stimulated CCD-18Co cells via inhibition of inflammatory proteins. QRT-PCR and western blotting data showed that LPS significantly increased the level of HIF-α, which was notably decreased in the presence of AS II (Figure 2A-C). In addition, the expressions of pro-inflammatory proteins p-p65 and p-IκB were detected by western blotting. As indicated in Figure 2B, 2D and 2E, the levels of p-p65 and p-IκB were markedly increased in LPS-stimulated CCD-18Co cells, compared with control group. However, LPS-induced p-p65 and p-IκB proteins upregulation were notably alleviated by AS II treatment. All these results suggested that AS II exhibited anti-inflammation effect in LPS-stimulated CCD-18Co cells via inhibition of inflammatory proteins.

**AS II alleviated the symptom of DSS-induced UC in mice**

A mouse model of DSS-induced UC was established to further investigate the anti-inflammation effect of AS II in vivo. As illustrated in Figure 3A and 3B, the body weights of mice were notably decreased at 10 days in AS II_80 mg/kg group, compared with control group. Meanwhile, 30 or 50 mg/kg AS II had very limited system toxicities. Therefore, AS II (30 and 50 mg/kg) was utilized in the following in vivo experiments.

H&E staining data showed that the infiltration of inflammatory cells and damage to the surface epithelium were observed in the DSS gro-
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However, preventive effect of AS II on DSS-induced UC was observed in DSS + AS II (30 or 50 mg/kg) group (Figure 3C). In addition, 50 mg/kg AS II markedly prevented the body weight loss in DSS-treated mice (Figure 3D). As we know, the severity of weight loss, stool consistency and blood in stool were reflected by DAI. As indicated in Figure 3E, 30 or 50 mg/kg AS II significantly reduced the DAI caused by DSS in mice, compared with the DSS group. Meanwhile, 50 mg/kg AS II significantly increased colon length during the progression of experimental UC in mice (Figure 3F). These data suggested that AS II could alleviate the symptom of DSS-induced UC in mice.

**AS II attenuated oxidative stress damage in DSS-induced UC mice via decreasing the levels of inflammatory factors**

Next, the levels of inflammatory cytokines in DSS-induced UC mice was detected by ELISA. As illustrated in Figure 4A-C, the levels of pro-inflammatory cytokines IL-6, TNF-α and IL-1β in colon tissues were markedly increased in the DSS group, compared with control group. However, DSS-induced IL-6, TNF-α and IL-1β upregulations were significantly alleviated by AS II treatment. In addition, the contents of NO, MDA and MPO were increased, and SOD activity was markedly reduced in colon tissues of DSS treated mice, which were markedly reversed by AS II treatment (Figure 4D-G). These data indicated that AS II could decrease the levels of inflammatory factors and attenuate oxidative stress damage in DSS-treated mice.

**AS II decreased the expression of inflammatory proteins in DSS-treated mice**

It is reported that NF-κB plays an important role in the mediation of inflammation in UC [20]. We next explored the effects of AS II on NF-κB pathway. IHC results indicated that the level of p-p65 was significantly increased in DSS-treated mice, which was markedly reduced by AS II treatment (Figure 5A). In addition, western blotting results showed the expressions of HIF-α, p-p65 and p-IκB were significantly upregulated in the DSS group. However, DSS-induced upregulations of HIF-α, p-p65 and p-IκB were significantly reversed by AS II treatment (Figure 5B-E). All these data illustrated that AS II could decrease the expressions of inflammatory proteins in DSS-treated mice.

**Discussion**

In this study, we investigated the mechanisms by which AS II repaired ulcerative colitis barrier function in vitro and in vivo. Based on in vitro and in vivo results, we found AS II significantly
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**Figure 4.** AS II attenuated oxidative stress damage in DSS-induced UC mice via decreasing the levels of inflammatory factors. The mice were treated with DSS, DSS and 30 mg/kg AS II or DSS plus 50 mg/kg AS II for 0, 2, 4, 6, 8 and 10 day. A-C. The levels of IL-6, TNF-α or IL-β in the colon tissues were measured with ELISA. D-G. The levels of NO, MPO, SOD and MDA in colon tissues were measured with ELISA. **P<0.01 compared with control group; *P<0.05, **P<0.01 compared with DSS group.

**Figure 5.** AS II inhibited the expression of inflammatory proteins in DSS-induced UC mice. The mice were treated with DSS, DSS and 30 mg/kg AS II or DSS and 50 mg/kg AS II for 0, 2, 4, 6, 8 and 10 day. A. The level of p-p65 in

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Alleviated LPS-induced inflammatory responses on Caco-2 cells and markedly reduced the production of inflammatory proteins in DSS-treated mice.

AS II is cycloartane-type triterpene glycosides, which has been reported to promote intestinal epithelial repair [9]. In present study, 3 μM AS II significantly inhibited the proliferation of Caco-2 cells, while the effect of 1 μM AS II on cell proliferation was extremely limited. These data illustrated that 1 μM was an appropriate concentration. In order to investigate the mechanisms responsible for the anti-inflammatory activities of AS II, LPS was added into Caco-2 cells to create an in vitro model of UC. LPS increased the production of pro-inflammatory cytokines IL-6, TNF-α and IL-β, while AS II markedly decreased the production of inflammatory factors in LPS-stimulated Caco-2 cells. Ke et al found that a traditional Chinese formulation (Qing Hua Chang Yin) markedly suppressed the secretion of IL-6 in LPS-stimulated Caco-2 cells [21]. These data suggested that AS II could decrease the secretion of inflammatory factors in LPS-stimulated Caco-2 cells.

In addition, dextran sulfate sodium (DSS)-induced UC mice model was used to further investigate the anti-UC effects of AS II. In this study, higher levels of pro-inflammatory factors TNF-α, IL-6, IL-1β were observed in the DSS group. However, AS II markedly alleviated DSS-induced production of pro-inflammatory factors. Moreover, MPO and NO also play important role in local intestinal damage [22, 23]. Meanwhile, SOD is an antioxidant enzyme that can reduce harmful substances produced during metabolism [24]. However, MDA is the product of lipid peroxidation, which reflect the severity of membrane damage in vivo [25]. In this study, we found that DSS-induced MPO, NO and MDA activity increases and SOD activity decreases in colon tissues were significantly reversed by AS II treatment. These data suggested that AS II could alleviate DSS-induced colonic injury via decreasing the production of inflammatory factors and attenuating oxidative stress damage. The above results strongly suggested that AS II alleviated the symptoms of UC in vitro and in vivo.

Hypoxia inducible factor-α (HIF-α) is a master regulatory transcription factor, which plays a vital role in regulating the inflammatory response [26]. When inflammation was appeared in cells or tissues, it would lead to inflammation-associated tissue hypoxia. Then, the level of HIF-α was increased in hypoxic cells or tissues [27]. Cristina et al found that HIF-α could induce the production of NO [28]. In addition, previous study indicated that HIF-α could induce inflammatory response via phosphorylation of IκB and NF-κB p-65 [29]. Meanwhile, large amount of NO could induce the activation of NF-κB [30]. NF-κB is a transcription factor, which could regulate the intestinal inflammatory response [31]. Various inflammatory factors, such as TNF-α, IL-6 and IL-12, have been shown to be regulated by NF-κB p-65 [32]. In this study, the levels of HIF-α, NO, p65 and IκB were markedly increased in colon tissues in DSS-induced UC mice, which were markedly decreased in the presence of AS II. Zhou et al indicated that Brusatol could alleviates the symptoms of UC via suppression of NF-κB-mediated inflammatory responses [33]. In addition, Ke et al found that Qing Hua Chang Yin attenuated LPS-induced inflammatory response in Caco-2 cells via suppressing the level of NF-κB [34]. These evidences illustrated that AS II could alleviate the symptoms of UC via inhibiting the NF-κB signaling. Hypoxia could occur under inflammation situation, which could activate HIF-α [35]. Meanwhile, HIF-α could activate NF-κB and pro-inflammatory cytokines in hypoxia, then hypoxia and inflammation are linked [36]. For the first time, we found that AS II could attenuate LPS-induced inflammatory response in Caco-2 cells via suppressing the level of HIF-α and then inactivating NF-κB. Therefore, our evidence suggests that AS II alleviated the symptoms of UC via inhibiting the HIF-α/NF-κB pathway.

Conclusion

In summary, AS II could alleviate the symptoms of experimental UC in vitro and in vivo via inhibition of HIF-α/NF-κB pathway. The evidences suggested that AS II may act as a potential agent for the treatment of UC.
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Disclosure of conflict of interest

None.

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