Ursolic acid protects against ulcerative colitis via anti-inflammatory and antioxidant effects in mice

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Abstract. Ursolic acid (UA) has been reported to have a protective effect in colitis. However, the underlying mechanisms remain to be elucidated. In the present study, experimental ulcerative colitis was induced in male BALB/c mice by the administration of 5% dextran sulfate sodium (DSS) for 7 days, followed by treatment with UA for another 7 days. Hematoxylin & eosin staining was performed to evaluate colon tissue damage, and enzyme assays were used to measure malondialdehyde (MDA) content and superoxide dismutase (SOD) activity in colon homogenate. In addition, serum levels of interleukin (IL)-1β and tumor necrosis factor (TNF)-α were measured using an ELISA, and the level of nuclear factor (NF)-κB p65 in the colonic tissues was assessed by western blotting. The 7-day DSS administration induced marked colon damage, increased the serum levels of IL-1β and TNF-α, increased MDA content and decreased SOD activity in the colon homogenate. These changes were significantly improved by treatment with UA. UA also reduced the DSS-stimulated high nuclear level of NF-κB p65 in the colon tissues. These results demonstrate a protective role of UA in ulcerative colitis, and suggest that anti-inflammatory and antioxidant activities are involved in the underlying mechanisms.

Introduction

Ulcerative colitis, a type of inflammatory bowel disease, is characterized by uncontrolled inflammation in the colon and rectum, recurrent episodes of bloody diarrhea, cramping, abdominal pain, mucosal inflammation and injury (1). Considering the pathogenic mechanisms of ulcerative colitis, it is suggested that deregulation of the pro-/anti-inflammatory systems and antioxidant system may to be involved (2). Currently, the medical treatment of ulcerative colitis relies predominantly on the use of traditional drugs, including aminosalicylates, corticosteroids and immunosuppressants. These conventional therapies for ulcerative colitis fail to successfully induce remission or prevent relapse, and can also cause various side effects (3). Therefore, there is an increasing interest in identifying alternative and more tolerable treatments for this disease.

Ursolic acid (UA), a natural pentacyclic triterpenoid carboxylic acid, is abundant in numerous plants, including apples, blueberries and hawthorn berries, and is the major component of certain oriental medicinal herbs, which are widely used in folk medicine (4). UA is well known to possess various biological activities, including anti-inflammatory (5,6), anticancer (7,8), hypoglycemic (9), antioxidant (10-12) and immunomodulatory activities (13). Previously, UA was reported to be involved in colitis in a number of publications (4,14,15). Hawthorn berry extract containing 0.5% UA decreased edema and the infiltration of neutrophils in the colonic tissues of acetic acid-treated rats (14). UA significantly inhibits the production of pro-inflammatory cytokines, including tumor necrosis factor (TNF)-α, interleukin (IL)-1β and IL-6, and the expression of cyclooxygenase-2 and inducible nitric oxide (NO) synthetase in lipopolysacharride (LPS)-stimulated macrophages and colonic tissues from 2,4,6-trinitrobenzenesulfonic-treated mice (4,15). UA also exerts an inhibitory effect on the activation of nuclear factor (NF)-κB in human intestinal epithelial cells and macrophages (4,15). These results indicate a protective role of UA in colitis. However, the detailed underlying mechanisms remain to be fully elucidated.

The present study aimed to investigate the protective effect of UA in a dextran sulfate sodium (DSS)-induced mouse model of ulcerative colitis, a widely used ulcerative colitis model (16), in order to elucidate the potential mechanisms underlying the anti-inflammatory and antioxidative activities of UA.

Materials and methods

Reagents. UA was purchased from Shanxi Huike Plant Development Co., Ltd. (Xi'an, China; purity ≥98%), dissolved in dimethyl-sulphoxide (DMSO; Sigma-Aldrich, St. Louis, MO, USA), and diluted in normal saline solution. The total

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quantity of DMSO did not exceed 1% upon assessment; a dose considered of no significance in the assays used. DSS was obtained from MP Biomedicals (Solon, OH, USA) and dissolved in distilled water. Chemiluminescence reagent was purchased from Pierce (Rockford, IL, USA). Rabbit anti-NF-κB p65 monoclonal antibody (cat. no. 4764) and rabbit anti-β-actin polyclonal antibody (cat. no. 4967) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit immunoglobulin (Ig)G secondary antibody (cat. no. sc-2004) was purchased from Santa Cruz Biotechnology, Inc. (Dallas, Texas, USA).

Animals. A total of 36 male BALB/c mice (7-week-old; 18-22 g) were obtained from the Experiment Animal Center, Liaoning Medical University (Jinzhou, China). The mice were allowed to adapt to the laboratory environment for 1 week prior to experiments. The animals were housed in plastic cages containing corn chip bedding, and were maintained on a 12-h light-dark cycle (07:00-19:00 h light cycle; 19:00-07:00 h dark cycle) with a room temperature of 22±1°C and a humidity of 65-70%. Water and food were available *ad libitum*. The animal experiments were approved by the Ethical and Research Committee of Liaoning Medical University (Jinzhou, China).

Induction of ulcerative colitis and UA treatment. DSS was used to induce ulcerative colitis in the present study. DSS-induced colitis is characterized by the mucosal infiltration of inflammatory cells, epithelial injury and ulceration, which are similar to acute and chronic ulcerative colitis in humans (17). In the present study, the mice were divided into four groups, as follows (9 mice/group): Control, DSS, DSS+UA and DSS+sulfasalazine [SFZ; a reference drug in colitis treatment (2)]. Colitis was induced by the provision of distilled water containing 5% DSS (w/v) for 7 days *ad libitum*, followed by fresh water. The mice in the DSS+UA and DSS+SFZ groups were then treated with UA (20 mg/kg) or SFZ (100 mg/kg), respectively, by gavage once per day for another 7 days. The mice in the DSS group were provided with the same volume of saline by gavage. The mice were assessed daily for behavior, body weight, presence of blood in stools and stool consistency.

Evaluation of disease activity index (DAI). The DAI was calculated for each animal, as reported previously (18). Briefly, the DAI was determined by combining the scores assigned for body weight loss, stool consistency and stool blood, with the mean of the three values deemed the DAI. A higher DAI score is indicative of more severe colon damage. Body weight loss was calculated as the percentage difference between the initial body weight and the final body weight. Stool blood was determined using the Fecal Occult Blood Test kit (Baso Diagnostics Inc., Zhuhai, China). The parameters investigated for the DAI evaluation are listed in Table I.

Measurement of serum IL-1β and TNF-α. Blood samples (0.5 ml/mouse) were collected from all mice following anesthetization with 0.1 ml phenobarbital (Sigma-Aldrich) and sacrifice by decapitation. Serum was collected following centrifugation at 2,000 x g for 10 min at 4°C and aliquoted. The aliquots (150 μl) were stored at -20°C until use. The serum

levels of IL-1β and TNF-α were measured using ELISA kits (cat. nos. EK0394 and EK0527, respectively; Wuhan Boster Biological Engineering Co., Ltd., Wuhan, China), according to the manufacturer's protocol.

Assessment of pathological damage. For histological analysis, the colons following collection of blood samples and rinsed with phosphate-buffered saline (PBS; pH 7.4). From each colon, two 2-cm long segments were removed from the proximal and distal ends, which were fixed in 4% paraformaldehyde (Sigma-Aldrich), dehydrated and embedded in paraffin (Sigma-Aldrich). These colon segments were cut longitudinally into 4-μm thick sections, which were then stained with hematoxylin and eosin (H&E; Leica Microsystems, Inc., Buffalo Grove, IL, USA). The colon sections were interpreted according to the criteria listed in Table II. The final colon pathological score was calculated as the average of the scores from the proximal and distal segments of the mouse colons.

Measurement of malondialdehyde (MDA) content, and superoxide dismutase (SOD) and myeloperoxidase (MPO) activities. The transverse colon tissues were rinsed and weighed, and were then placed into tubes with nine volumes of normal saline. The tissue samples were then homogenized. Following centrifugation at 3,000 x g for 10 min at 4°C, the MDA content, and activities of SOD and MPO in the supernatant, were measured using commercially available kits (cat. nos. A003, A001 and A044, respectively; Nanjing Jiancheng Bioengineering Institute, Nanjing, China), according to the manufacturer's protocol.

Western blotting for the protein expression of NF-κB p65. The nuclear proteins in the transverse colon tissues were extracted using a kit, according to the manufacturer's protocol (Wuhan Boster Biological Engineering Co., Ltd.). The protein concentration was quantified using a bicinchoninic acid assay (Thermo Fisher Scientific, Inc., Waltham, MA, USA). The nuclear protein expression of NF-κB p65 was measured using Western blotting, in accordance with a previous report (19). Briefly, the nuclear proteins (20 μg) were separated electrophoretically on 4-12% SDS-PAGE gels, and transferred onto nitrocellulose membranes (Thermo Fisher Scientific, Inc.). Following 30 min of blocking with 2.5% nonfat milk, the membranes were incubated with rabbit anti-NF-κB p65 monoclonal antibody (1:1,000) and rabbit anti-β-actin polyclonal antibody (1:2,000) at 4°C overnight, followed by 1 h incubation with HRP-conjugated goat anti-rabbit IgG (1:2,000). The membranes were washed with PBS containing 0.5% Tween 20 (Sigma-Aldrich) subsequent to each antibody treatment. The membranes were developed using chemiluminescence reagent and then exposed to X-ray film (Midwest Scientific, Valley Park, MO, USA). The band intensities were analyzed using ImageJ software, version 1.5g (https://imagej.nih.gov/ij/). The protein levels of p65 are expressed as the ratio of the band optical intensity to that of β-actin.

Statistical analysis. Data are presented as the mean ± standard error of the mean. Statistical analysis was performed using SPSS 17.0 statistical software (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance and Dunnett's test were used

Table I. Parameters of disease activity index evaluation.

Score	Weight loss (%)	Stool consistency	Blood in stool
0	-	Normal	-
1	1-5	Normal	+
2	6-10	Very soft but formed	++
3	11-15	Liquid	+++
4	>15	Liquid	Gross rectal bleeding

Table II. Pathological scores of colitis in colon tissues.

| Score	Pathological parameter
0	Normal tissues
1	Mild inflammation in the mucosa with some infiltrating mononuclear cells
2	Increased level of inflammation in the mucosa with more infiltrating cells, crypt glands pulled away from basement membrane, mucin depletion from goblet cells, epithelium beginning to pull away from mucosa into lumen
3	Extensive infiltrating cells in the mucosa and submucosa area, crypt abscesses present, increased mucin depletion, epithelial cell disruption
4	Extensive infiltrating cells in the tissue, complete loss of crypts
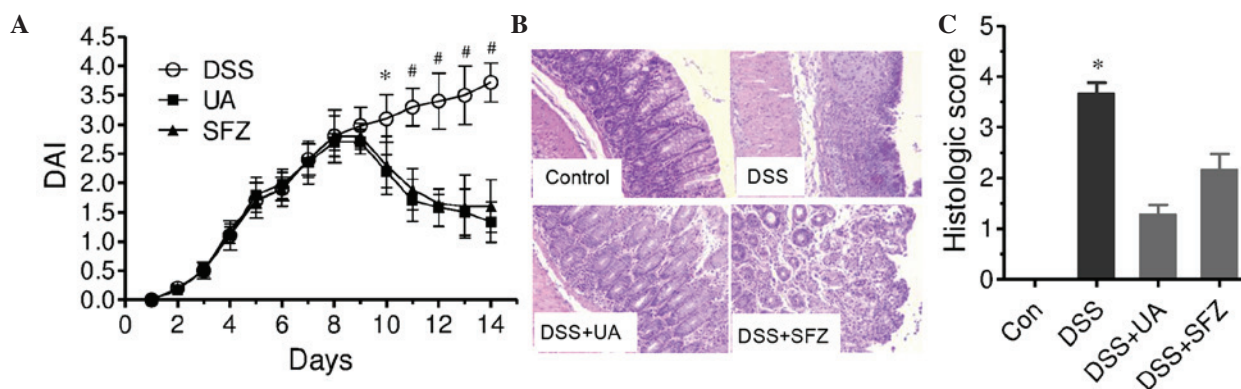
Figure 1. Effects of UA on DAI and histological injury. (A) Effects of UA on DAI. Data are presented as the mean ± standard error of the mean. *P<0.05, #P<0.01 vs. the UA or SFZ group. (B) Histological changes in the colon were detected following hematoxylin and eosin staining (magnification, x200). (C) Pathological scores following administration of DSS for 7 days, followed by treatment with UA (20 mg/kg) or SFZ (100 mg/kg) for 7 days (n=6-9). Data are presented as the mean ± standard error of the mean. *P<0.05 vs. the control, UA and SFZ groups. DSS, dextran sulfate sodium; UA, ursolic acid SFZ, sulfasalazine; DAI, disease activity index.

for group comparisons. P<0.05 was considered to indicate a statistically different difference.

Results

Effects of UA on DAI and histological injury. The administration of 5% DSS in mice for 7 days resulted in marked clinical and histological signs of colitis. These mice exhibited loose stools or diarrhea, occult or gross rectal bleeding, and weight loss. The DAI score of colitis in the DSS-treated mice was significantly increased with time, and increased continuously, even following the termination of DSS treatment (Fig. 1A). Following 7 days of DSS administration, the mice

were treated with UA (20 mg/kg daily) for another 7 days. UA treatment significantly reduced DSS-induced DAI following 3 days of treatment with UA (day 10), and this reduction was more marked following 7 days of treatment (P<0.05; Fig 1A). SFZ, a well-known prodrug, which releases 5-amino-salicylic acid in the intestine to disturb the prostaglandin and leukotriene pathways, has been the most widely-prescribed anti-colitis drug (2). As a reference drug, SFZ (100 mg/kg daily) also significantly decreased the DSS-induced DAI in the present study (P<0.05; Fig 1A).

The administration of DSS in the mice for 7 days caused marked colon tissue damage. H&E staining of the colon tissues exhibited extensive infiltrating cells, epithelial cell disruption
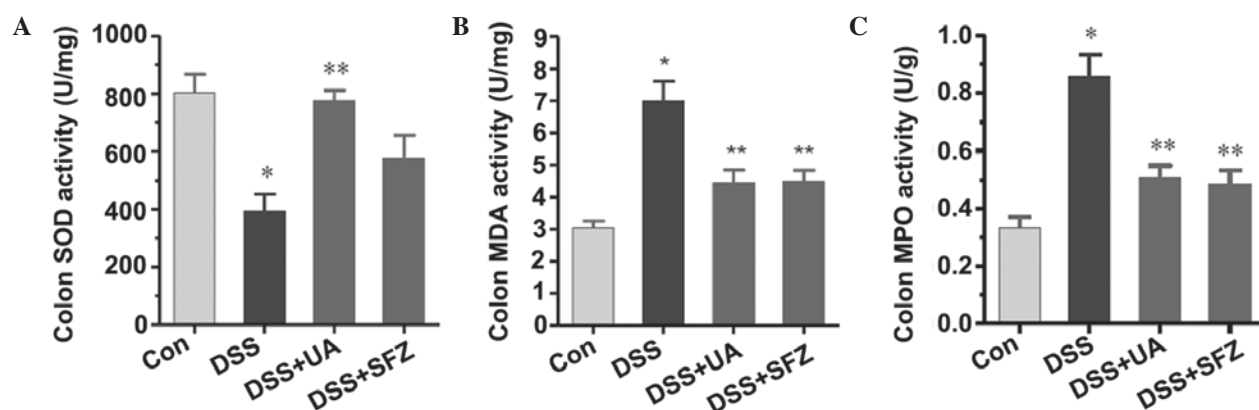
Figure 2. Effects of UA on MDA content, and SOD and MPO activities in colon tissues. The mice were treated with DSS for 7 days, and then with UA or SFZ for a further 7 days. Colon homogenates were used to measure (A) SOD activity, (B) MDA content and (C) MPO activity. The results are expressed as MDA content, and SOD and MPO activity per mg colon tissue, as the mean ± standard error of the mean (n=6-9). *P<0.05, vs. Con group; **P<0.05, vs. DSS group. UA, ursolic acid; DSS, dextran sulfate sodium; SFZ, sulfasalazine; MDA, malondialdehyde; SOD, superoxide dismutase; MPO; myeloperoxidase.
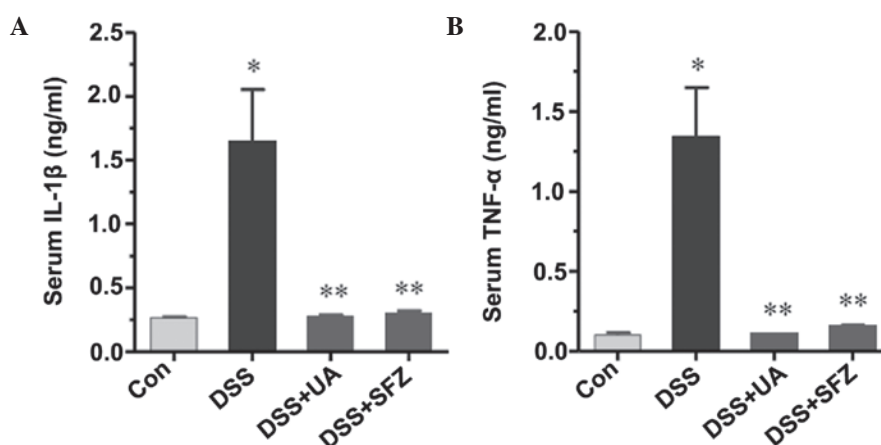

Figure 3. Effects of UA on serum levels of IL-1β and TNF-α. The mice were treated with DSS for 7 days and then with UA or SFZ for a further 7 days. The serum levels of (A) IL-1β and (B) TNF-α were measured using an enzyme-linked immunosorbent assay. Data are presented as the mean ± standard error of the mean (n=6-9). *P<0.01, vs. Con group; **P<0.05, vs. DSS group. UA, ursolic acid; DSS, dextran sulfate sodium; SFZ, sulfasalazine; Con, control; IL-1β, interleukin-1β; TNF-α, tumor necrosis factor-α.

or the complete loss of crypts in the DSS group (Fig. 1B). In the DSS group, the histopathological score was increased to 3.67, which was significantly increased, as compared with the score of 0 in the control group (P<0.05; Fig. 1C). UA treatment significantly improved the DSS-induced colon histological injury, and decreased the pathological score to 1.29 (P<0.05). The reference drug, SFZ, also improved histological injury, with a pathological score of 2.17 (P<0.05, vs. DSS group). The results from the DAI and pathological examinations demonstrated the protective role of UA in colitis.

UA decreases MDA content and MPO activity, and increases SOD activity in colon tissues. The present study then examined the possible mechanisms by which UA offers protection from colitis. As UA exhibits antioxidant activity in certain cell models or cell-free models (10-12), the present study aimed to determine whether UA interferes in antioxidant ability in colon tissues. The content of MDA, a marker of lipid peroxidative damage, and activity of SOD, an important anti-oxidative enzyme were measured. As shown in Fig. 2A and B, DSS administration significantly decreased SOD activity and

increased MDA content in the colon tissues, compared with the control group (P<0.05). Treatment with UA significantly reversed the DSS-induced changes in SOD activity and MDA content (P<0.05). SFZ also induced a significant decrease in DSS-stimulated MDA content, and a non-significant increase in SOD activity. Hawthorn berry extract, a source of UA, has been found to decrease neutrophil infiltration in colon tissues in a colitis model (14). In the present study, MPO activity, an important maker of neutrophils, was measured to examine the effect of UA on neutrophil infiltration. As shown in Fig. 2C, UA significantly decreased colonic MPO activity stimulated by DSS administration. Simultaneously, MPO activity was also reduced by SFZ treatment.

UA decreases the expression of pro-inflammatory cytokines. DSS induced a marked inflammatory response in the colon tissues, which was significantly improved by UA treatment. Thus, the present study examined whether UA decreases the levels of pro-inflammatory cytokines. The levels of IL-1β and TNF-α cytokines were measured using ELISA. As shown in Fig. 3, DSS administration significantly increased
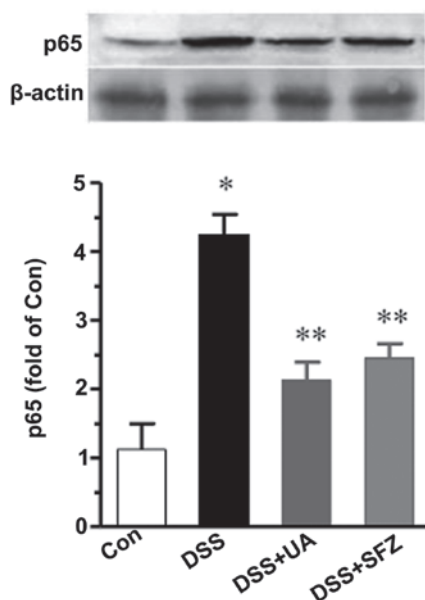
Figure 4. Effects of UA on the nuclear levels of nuclear factor-κB p65 in colon tissues. The mice were treated with DSS for 7 days, and then with UA or SFZ for a further 7 days. The nuclear protein in the colon tissues was used to measure the levels of p65 using Western blotting. Data are presented as the mean ± standard error of the mean (n=5). *$P<0.01$, vs. Con group; **$P<0.05$, vs. DSS group. UA, ursolic acid; DSS, dextran sulfate sodium; SFZ, sulfasalazine; Con, control.

the serum level of IL-1β, compared with the control group ($P<0.01$). This increase in the level of IL-1β was significantly decreased by UA or SFZ treatment ($P<0.05$). Similar to the results of IL-1β, DSS administration increased the serum level of TNF-α, which was also significantly decreased by UA or SFZ treatment.

UA decreases the level of NF-κB p65. The expression levels of pro-inflammatory factors, including IL-1β and TNF-α is closely regulated by transcription factor activity, particularly NF-κB. Thus, the present study examined whether UA alters the activity of NF-κB in colon tissues using Western blotting. As shown in Fig. 4, the nuclear level of NF-κB p65 was markedly increased by DSS, compared with the control group ($P<0.01$). Treatment with UA or SFZ resulted in a significant reduction of p65 ($P<0.05$).

Discussion

The present study aimed to evaluate the role of UA in DSS-induced colitis and the underlying mechanisms. The results showed that UA significantly improved DSS-induced colitis in mice, which was shown by improvements in body weight, stool consistency, rectal bleeding and pathologic involvement of colon tissues, and a decrease in neutrophils infiltration reflected by MPO activity. UA also decreased serum levels of IL-1β and TNF-α, decreased MDA content and increased SOD activity in colon. Finally, UA downregulated DSS-stimulated nuclear expression of transcription factor NF-κB. These results demonstrated a protective role of UA in experimental colitis. Antioxidant and anti-inflammatory activity may underlie the mechanisms of action.

Reactive oxygen species (ROS) is an important factor involved in ulcerative colitis. Oxidative stress and its consequent lipid peroxidation can aggravate free radical chain reactions, disrupt the integrity of intestinal mucosa barrier and activate pro-inflammatory responses. MDA, a marker of lipid peroxidative damage, and SOD, an important antioxidative enzyme, have been reported to increase and decrease in experimental colitis studies, respectively (20,21). Antioxidative and anti-inflammatory treatment improved these effects on the MDA level and SOD activity in experimental ulcerative colitis (20,21).

UA has showed beneficial effects on SOD activity and MDA content in various cells and animal models. UA pretreatment significantly reverses H_2O_2- and 1-Methyl-4-phenylpyridinium-induced impairment in SOD activity, and decreases MDA formation in PC12 glial cells (22). UA increases SOD in Ehrlich ascites carcinoma tumor (23). UA has also demonstrated a neuroprotective effect on D-gal induced neurotoxicity in mice via, at least in part, increases in the activity of SOD enzymes with a reduction in MDA content (24). UA has also shown a protective effect in chronic ethanol-induced oxidative stress in the rat heart via the improvement of SOD activity (25). In the present study, SOD activity was decreased and MDA content was increased in the colon tissues of the DSS group, compared with those of the control group. This indicated that DSS administration damaged the antioxidative ability, which may subsequently cause the onset of colitis onset. This is consistent with previous reports (26,27). In the present study, UA treatment for 7 days significantly improved the increased MDA content and decreased SOD activity in the colon tissues. These results suggested that antioxidative protection may be an important mechanism for UA in colitis. Furthermore, the accumulation of ROS in colon tissues, in addition to directly damaging intestinal epithelial cells, stimulates inflammatory responses to release pro-inflammatory cytokines, particularly TNF-α and IL-1β (28,29), which may cause further damage.

Ulcerative colitis is a type of inflammatory bowel disease. It is characterized by pro-inflammatory cell infiltration and pro-inflammatory cytokine release. Several cytokines, including TNF-α, IL-1β and inducible NO synthetase are crucial components of these inflammatory pathways (30). In the present study, the levels of TNF-α and IL-1β were markedly upregulated during the onset of DSS-induced colitis induction. Treatment of ulcerative colitis commonly reduces inflammatory cell infiltration and the levels of serum TNF-α and IL-1β (31,32). In particular, treatment with TNF-α neutralizing antibody or TNF-α inhibitory agents significantly improveds colitis (33,34). Therefore, suppression of these cytokines can offer an alternative therapy for ulcerative colitis. In the present study, when UA was applied in the DSS-administrated mice, the serum levels of TNF-α and IL-1β were significantly decreased. Given the improvement of clinical features and pathological features by UA, UA may act an inflammation alleviator in the treatment of ulcerative colitis. Of note, UA has also been found to decrease various pro-inflammatory markers, including TNF-α, IL-1β, IL-6, IL-17 and cyclooxygenase-2, and ameliorate inflammatory injury in experimental nephritis and hepatitis models (35,36).

In the present study, it was also demonstrated that UA significantly decreased DSS-stimulated nuclear levels of NF-κB p65 in colon tissues. NF-κB is a dimeric transcription factor containing p50 and p65 subunits, and p65 nuclear translocation represents the activation state of NF-κB. NF-κB activates the expression of several genes involved in inflammatory process, including IL-1β and TNF-α. In earlier studies, UA showed a suppressive effect on NF-κB activity in several disease models. UA protects against carbon tetrachloride-induced injury in the kidney and liver via inhibition of NF-κB activities (35,36). UA also inhibits T cell activation and proliferation via inhibition of the NF-κB signaling pathway (37). The administration of UA also decreases the product of lipid peroxidation and decreases the expression of NF-κB following stroke in mice (38). UA also markedly inhibits serum levels of TNF-α, IL-6 and IL-1β, decrease MAD levels and attenuates the protein expression of NF-κB in the lungs of LPS-treated mice (39). In terms of the gastrointestinal system, UA has a direct inhibitory effect on NF-κB activation in intestinal epithelial cells stimulated by TNF-α, and in peritoneal macrophages stimulated by LPS (4). UA also suppresses NF-κB signaling in colon cancer cells (7), and other types of cancer cell (40,41). In the present study, the nuclear level of NF-κB p65 in the colon tissues of the DSS group was significantly increased, compared with that of the control group. The increased nuclear level of NF-κB p65 was significantly reversed by UA treatment. This result, together with the direct inhibitory effects of UA on NF-κB signaling in intestinal epithelial cells and colon cancer cells described above, indicated that NF-κB signaling system may be important in the induction of colitis, and act an important mechanism in the protective effects of UA. Although the cause or subsequent effects of NF-κB suppression and inflammation improvement remains to be fully elucidated, UA treatment not only decreased the nuclear expression of NF-κB, but also downregulated the production of IL-1β and TNF-α, and thereby ameliorated the severity of the colitis. Therefore, supplementation with UA may be an efficacious and promising agent in the treatment of ulcerative colitis.

In conclusion, the present results demonstrated that UA exerted a beneficial effect on DSS-induced colitis in mice. As a possible mechanism, UA improved the SOD activity, increased scavenging of oxidative-free radicals, and downregulated the levels of cytokines, including TNF-α and IL-1β. The suppression of the NF-κB transcription system may underlie the mechanism of the anti-inflammatory ability of UA.

Acknowledgements

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