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Suppression of MAPK and NF-κ B pathways by schisandrin B contributes to attenuation of DSS-induced mice model of inflammatory bowel disease

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Received February 24, 2015, accepted March 23, 2015

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Pharmazie 70: 598–603 (2015) *doi: 10.1691/ph.2015.5561*

Schisandrin B (*Sch B*), the most abundant dibenzocyclooctadiene lignan isolated from the traditional Chinese medicinal herb Schisandra chinensis (Turcz.) Baill, possesses various biological activities, such as hepatic protection, anti-tumor, anti-inflammatory and anti-cardiovascular properties. However, the effect of *Sch B* on inflammatory bowel disease (IBD) is not yet known. The aim of this study was to investigate whether *Sch B* has protective effect against dextran sulfate sodium (DSS)-induced colitis in a mouse model. The acute mouse model of IBD was induced by drinking 2.5% DSS water for 5 days. *Sch B* was administered orally in doses of 10, 40, and 100 mg/kg respectively. It significantly reduced concentration of TNF-α, IL-1β, INF-γ and IL-6 in colon tissue as well as the mRNA expression levels. In addition, we demonstrated that *Sch B* blocked the phosphorylation of IκBα, nuclear factor-κB (NF-κB) p65, p38 mitogen-activated protein kinase (MAPK), c-Jun NH2-terminal kinase, and extracellular signal regulated kinase in DSS-induced acute colitis. In conclusion, these results indicated that *Sch B* could exert beneficial effects on experimental IBD induced by DSS and may represent a novel treatment strategy for IBD.

1. Introduction

Inflammatory bowel disease (IBD) including Crohn's disease (CD) and ulcerative colitis (UC) is characterized by mucosal damage of the intestine which is caused by aberrant innate and/or adaptive immune responses. It results from a complex interplay of external factors and genetic susceptibility and immune system dysfunction (Fiocchi 1998). Twin studies and epidemiologic studies showing a familial and ethnic clustering of the IBDs were the first indicators for a significant genetic component in the development of these diseases (Wirtz and Neurath 2007).

Dextran sulfate sodium (DSS)-induced colitis is one of the most commonly used models. DSS colitis reflects many of the clinical features of UC. For example, changing the DSS concentration or administration cycles can easily induce acute, chronic or relapsing colitis. Moreover, the dysplasia that frequently occurs after the chronic phase of DSS colitis resembles the clinical course of human UC (Wirtz et al. 2007; Perše and Cerar 2012). Recent reports have focused on the multifunctional role of DSS for *in vivo* colitis modeling (Fazio et al. 2014). In the most widely used DSS murine model, animals are treated with 2.5% DSS in their drinking water for five days. This provides a model of acute intestinal injuries.

In the process, it has been reported that small molecules or antibodies targeting NF-κB and proinflammatory cytokines such as TNF-α, IFN-γ, IL-1β and IL-6 and their signaling pathways were highly effective in attenuating the inflammation-associated intestinal damages in IBD patients and a number of animal models of colitis (Scaldaferri and Fiocchi 2007). NF-κB, a primary regulator of inflammatory responses, plays a key role in mediating inflammation. It plays a central role during inflammatory responses and in autoimmune diseases. Additionally, recent evidence has also shown the Mitogen-activated proteinkinases (MAPK) are also involved in inflammation. MAPKs, which are a family of proteins, including p38, extracellular signal-regulated kinase (ERK), and c-jun NH2-terminal kinase (JNK), play a central role during inflammatory responses and in autoimmune diseases (Cargnello and Roux 2011). They are important players in signal transduction pathways activated by a range of stimuli and mediate a number of physiological and pathological changes in cell function. Treatments aimed at inhibition of NF-κB and MAPKs may have potential therapeutic advantages in curing inflammatory diseases.

Schisandrin B (*Sch B*, Fig. 1), the main active ingredient isolated from the fruit of Schisandra chinensis (Turcz.) Baill, is a lignan component (Zhang et al. 2014). *Sch B* has been extensively studied for its biological activities. It has been reported to protect the liver from hepatotoxicity induced carbon tetrachloride (Ip et al. 1995). Pharmacological studies of *Sch B* have also revealed anti-cancer, antioxidation and neuroprotective effects. In particular, *Sch B* exhibits anti-inflammatory activities both *in vitro* and *in vivo* (Sun et al. 2007; Chiu et al. 2008; Lee et al. 2012; Checker et al. 2012). Interestingly, protective effect of *Sch B* against intestinal inflammation in IBD has never been investigated.

We evaluated the protective effects of *Sch B* against DSS-induced colitis. Furthermore, possible mechanisms of these protective effects were investigated. The results of this study may be helpful in the discovery of new reagents to cure IBD. Our results might provide a pharmacological basis on its clinical use for treatment of IBD.

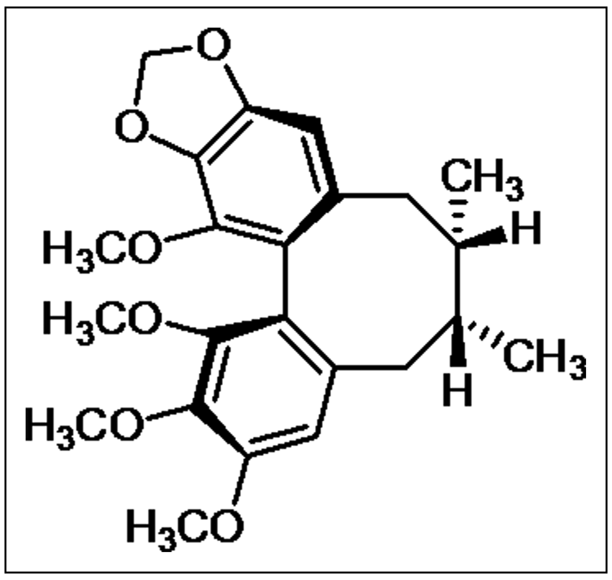

Fig. 1: Chemical structure of the schisandrin B.

2. Investigations and results

2.1. Histological evaluation of colitis

To investigate the effects of *Sch B* on the colon, hematoxylin-eosin staining (HE) was used. Histological evaluation of the colon was made from the colocecal junction to the anus. After 5 days of DSS treatment (Fig. 2B), histological signs of colonic inflammation in model group were multifocal mucosal infiltrations of predominantly neutrophils and lymphohistocytes multifocal submucosaloedema and pronounced loss of goblet cells. In contrast, *Sch B* treatment at high dose (100 mg/kg) significantly ameliorated the histological alterations induced by DSS as shown in the representative images (Fig. 2E).

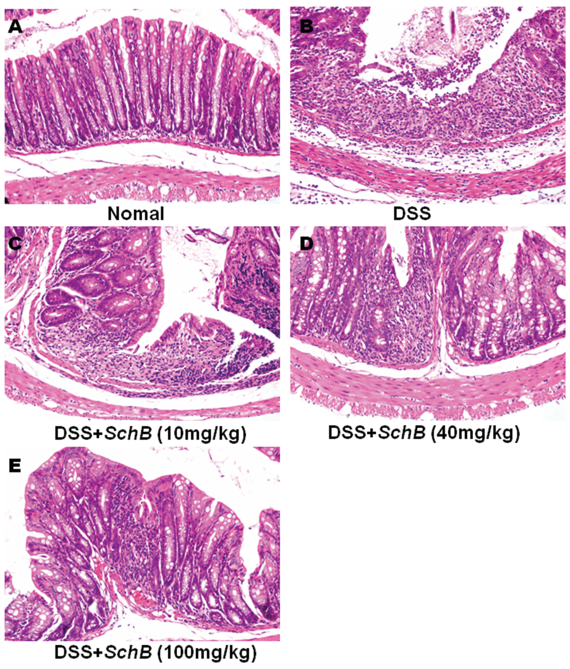

Fig. 2: Effect of Sch B on DSS-induced colitis histopathogic changes. Colons (n = 3) from each experimental group were processed for histological evaluation. Representative histological section of the colons was stained by hematoxylin and eosin (H&E staining, magnification × 100).

2.2. Sch B reduces the release of cytokines TNF-α, IL-1β, IFN-γ and IL-6

Compared with the control group, IL-1β, IFN-γ and IL-6 in homogenate of colon tissue significantly increased after DSS ($p < 0.01$). And the level of TNF-α ($p < 0.05$) was found to be increased in the DSS group compared with the normal group. *Sch B* (40 mg/kg, 100 mg/kg) can reduce the release of TNF-α, IL-1β, IFN-γ and IL-6 in a dose-dependent manner, respectively. And the levels of TNF-α, IL-1β, IFN-γ and IL-6 in the *Sch B* group were marked lower than that in the DSS group. All the results are shown in Fig. 3.

2.3. Sch B decreases the gene expression of cytokines TNF-α, IL-1β, IFN-γ and IL-6

TNF-α, IL-1β, IFN-γ and IL-6mRNA levels in the colon were measured by RT-PCR. As shown in Fig. 4, the mRNA levels of TNF-α, IL-1β, IFN-γ and IL-6 in DSS group increased significantly compared with those in control group. In *Sch B* (40 mg/kg, 100 mg/kg), the mRNA levels of TNF-α, IL-1β, IFN-γ and IL-6 were significantly decreased in a dose-dependent manner compared to the model group, respectively ($P < 0.05$).

2.4. Sch B inhibits the activation of NF-κB and MAPKs signal pathways

Activation of MAPK and NF-κB pathways, which play key roles in the regulation of inflammation, was determined as the ratio between the levels of phospho- and total ERK1/2, p38, and JNK kinases, and those of NF-κB inhibitor IκB-α, for MAPK and NF-κB pathways respectively. DSS stimulation resulted in prominent phosphor-IκBα, phosphor-NF-κB p65 (Fig. 5) and phosphorylation of MAKs (Fig. 6) in mouse colon tissue. The expression of p-NF-κB p65 protein was significantly decreased, which correlated with a similar decrease in p-IκB protein after *Sch B* treatment, especially at the dose of 75 mg/kg.

3. Discussion

Although the exact etiology of IBD has not yet been defined, it is clear that genetic factors, bacterial flora, and local mucosal host responses are important and that various proteins mediate the observed effects of disease (Chassaing and Darfeuille-Michaud 2011). It is a non-specific inflammatory disorder characterized by leukocyte infiltration, oxidative stress and upregulation of inflammatory mediators. DSS ingestion can provoke intestinal inflammation as well as clinical and pathological manifestations of colitis (Yousef et al. 2012). To evaluate the histological changes after *Sch B* administration in DSS-treated mice, colon sections were subjected to hematoxylin and eosin staining. As a result, dilapidation of tissues epithelium induced inflammatory infiltration. A very high level of leukocyte infiltration was observed in colon tissue by pathological examination in DSS-induced IBD.

A key component of the host innate immune response to injury is the up-regulation of cytokine production. The inflammatory responses are dynamic and sophisticatedly regulated by the cytokine network during an infection (Abraham and Medzhitov 2011). Among others, TNF-α, IL-1β, IFN-γ andIL-6 are known to be important inflammatory mediators released in response to many different injurious stimuli associated with worse outcome in patients with IBD (Oz and Ebersole 2008). They are involved in the initiation and development of acute inflammation. In particular, as the central inflammatory cytokine in the pathogenesis of IBD, anti-TNF-α therapy in patients of IBD

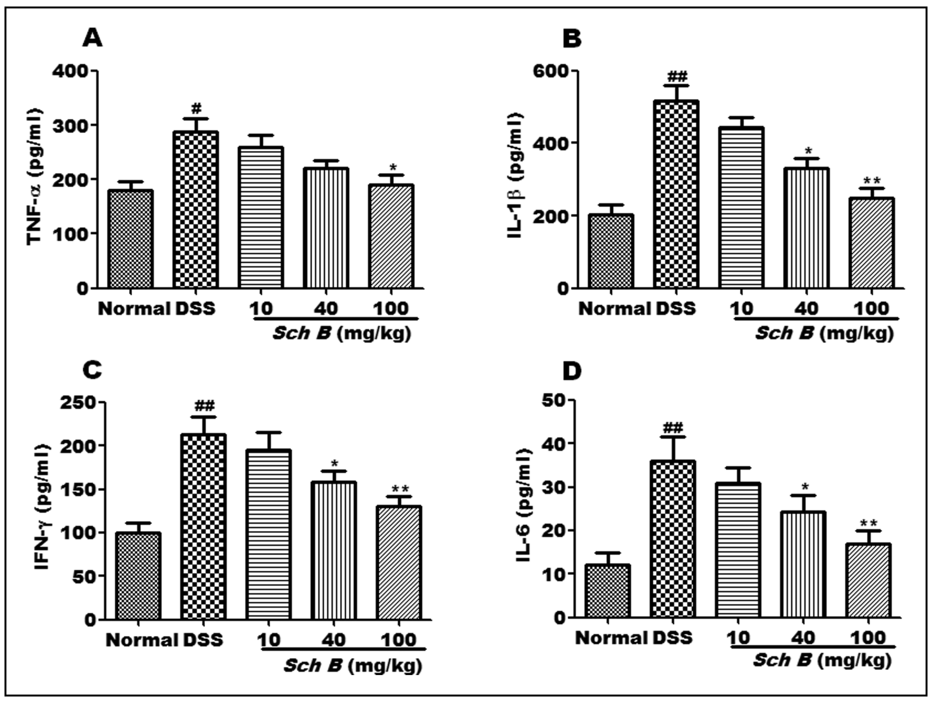

Fig. 3: Effect of Sch B on inflammatory cytokine production in colonic tissues. The levels of TNF-α, IL-1β, IFN-γ and IL-6 were measured by ELISA kits after 10 days treated. Data represent the means ± SEM in each group (n = 5), ##$p < 0.01$, ###$p < 0.001$ compared with the control group; *$p < 0.05$, **$p < 0.01$ compared with the model group.

has been proven effective (Rutgeerts et al. 2004). In the present study, the plasma levels of TNF-α, IL-1β, IFN-γ and IL-6 were significantly increased in DSS-treated mice compared to the normal mice. Administration of *Sch B* attenuated the production of TNF-α, IL-1β, IFN-γ and IL-6 in DSS-induced mouse colitis in a dose-dependent manner. *Sch B* also remarkably suppressed the transcription levels of TNF-α, IL-1β, IFN-γ and IL-6 in colon tissue compared to the DSS group. Therefore, *Sch B* may protect against DSS-induced mouse colitis by decreasing the transcription and production of these proinflammatory cytokines.

To further characterize the nature of the inhibitory effect of *Sch B* on cytokine production, we examined the effects of *Sch B* on activation of the NF-κB and MAPKs signaling pathways. NF-κB is the critical transcription factor needed to express genes associated with a proinflammatory response (Koo et al. 2010). It has been reported that NF-κB inhibitors have preventive and therapeutic effects for IBD in rats (Mizoguchi 2011). DSS markedly induces phosphorylation of NF-κB P65 and IκBα, and administration of *Sch B* impairs phosphorylation of these molecules in a dose-dependent manner. In addition, we examined the effects of *Sch B* on activation of MAPKs, which play a pivotal role during inflammatory responses. As shown by our experiments, DSS obviously raised the phosphorylation of p38, ERK, and JNK, which were down-regulated by intraperitoneal administration of *Sch B*. The result demonstrated that NF-κB and MAPKs were activated in DSS-induced mouse colitis, and more importantly, the inhibitory action of *Sch B* on the activation of NF-κB

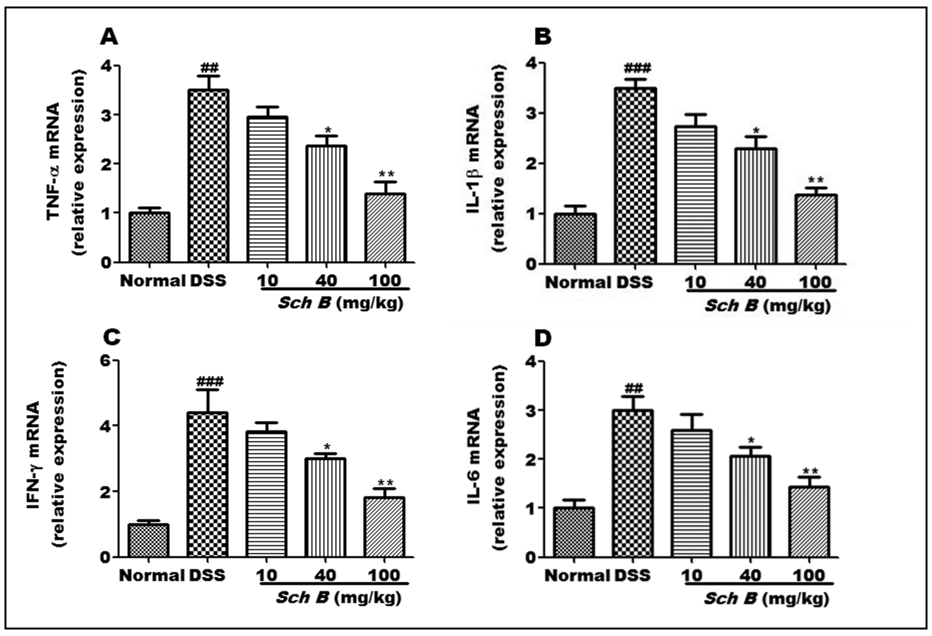

Fig. 4: The mRNA levels of TNF-α, IL-1β, IFN-γ and IL-6 in mouse colon after 10 days treated was detected by RT- PCR. Data represent the means ± SEM in each group (n = 5), #$p < 0.05$, ##$p < 0.01$ compared with the control group; *$p < 0.05$, **$p < 0.01$ compared with the model group.

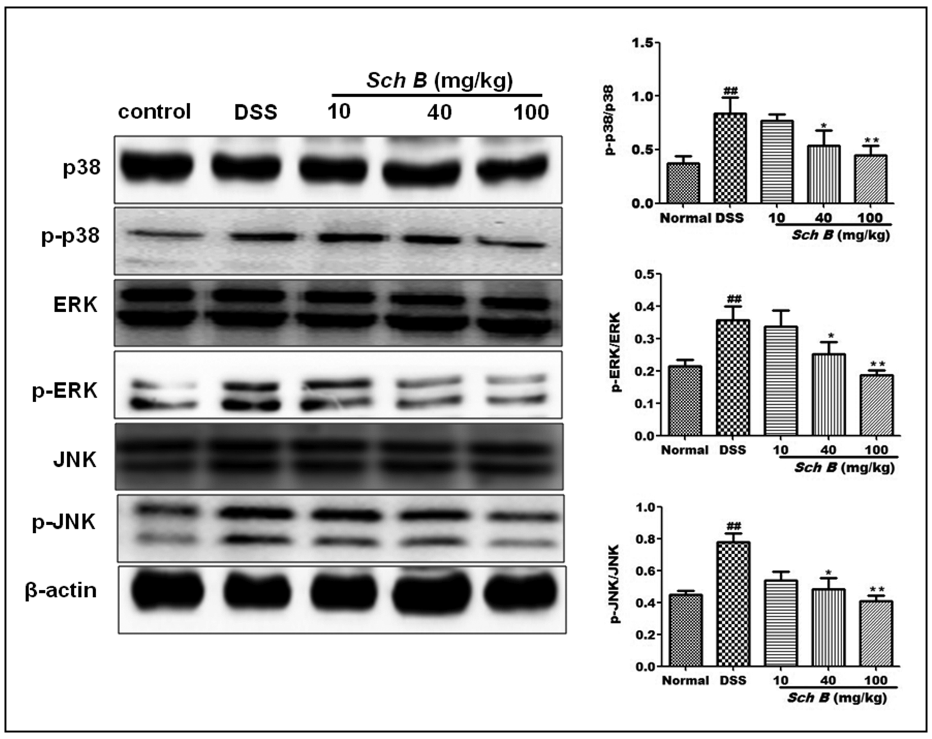

Fig. 5: Effect of Sch B on the activation of NF-κB in the colons of DSS-induced colitis mice. Protein samples were analyzed by western blot with specific antibodies as described. β-actin was used as an internal control. Similar results were obtained in three independent experiments and one of three representative experiments is shown. Data represent the means ± SEM in each group (n = 3), ###$p < 0.001$ compared with the control group; *$p < 0.05$, **$p < 0.01$ compared with the model group.

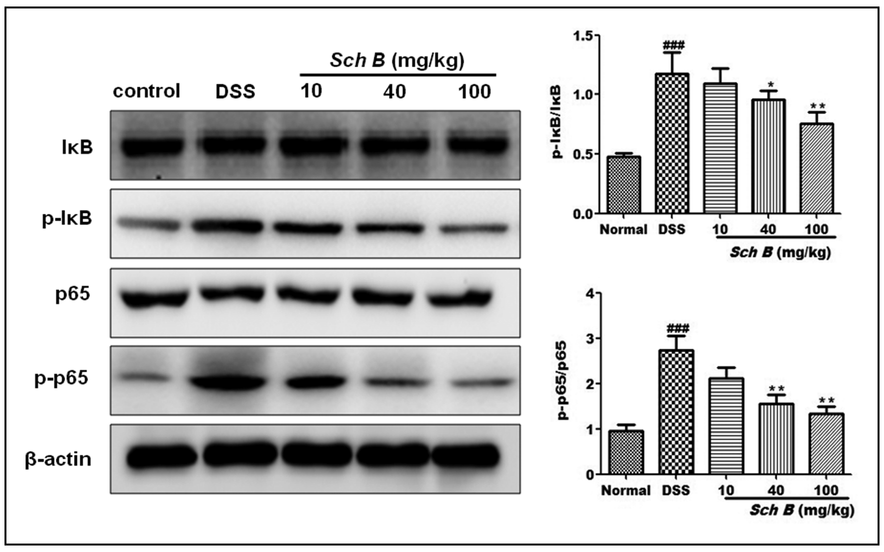

Fig. 6: Effect of Sch B on DSS-induced phosphorylation of ERK, JNK, and p38 MAPK in mouse colons. Protein samples were analyzed by western blot with specific antibodies as described. β-actin was used as an internal control. Similar results were obtained in three independent experiments and one of three representative experiments is shown. Data represent the means ± SEM in each group (n = 3), ##$p < 0.01$ compared with the control group; *$p < 0.05$, **$p < 0.01$ compared with the model group.

and MAPKs blocked the up-regulation of pro-inflammatory cytokines.

In the present study, we demonstrated for the first time that *Sch B* treatment can markedly reduce colon inflammation and histological tissue injury in the DSS-induced mouse model. This observation is supported by the results obtained from ELISA data and histological findings. Importantly, we found that *Sch B* attenuated proinflammatory cytokine production *via* inhibiting the activation of NF-κB and MAPKs signal pathways. Therefore, these data strongly suggest that *Sch B* has potent anti-inflammatory activity and might be used as a clinical drug to cure colitis. To explore the precise mechanism and the full effects of *Sch B*as a clinical agent to treat IBD, more work should be done.

In conclusion, studies from animal models have improved our understanding of the complexity of human IBD and allowed the molecular dissection of pathophysiological mechanisms that are presumably responsible for disease initiation and progression. The present study has demonstrated that *Sch B* significantly increased the release of the colon levels of TNF-α, IL-1β, IFN-γ and IL-6. *Sch B* has anti-inflammatory actions *via* suppressing NF-κB activation in DSS induced acute UC mice model. It also has been demonstracted that *Sch B* inhibited ERK, JNK, p38MAPK. These results indicate that *Sch B* has a potency as a new functional food for IBD patients. The effect of *Sch B*on human IBD should be carefully evaluated in the future.

4. Experimental

4.1. Chemicals and reagents

Schisandrin B (pure: 98%) was purchased from the National Institutes for Food and Drug Control (Beijing, China). DSS (molecular weight, 36-50 kDa; reagent grade) was purchased from MP Biomedicals LLC (Solon, OH, USA). The enzyme-linked immunosorbent assay (ELISA) kits for determination of IL-6, IL-1β, IFN-γ and TNF-α were produced by Nanjing KeyGEN Biotech. CO., LTD. (Nanjing, China). Primary antibodies for IκBα, p65, p-p65, p-38, p-p38, ERK, p-ERK, JNK, p-JNK and p-IκBα were purchased from Cell Signaling Technology Inc (Beverly, MA, USA). And secondary antibodies were acquired from Santa Cruz Biotechnology (Santa Cruz, CA, USA).

4.2. Animals

Adult female C57BL/6 mice, weighing 18-20 g, were provided by the Vital River Laboratory Animal Center (Beijing, China). Mice were housed with free access to food and water under a natural day/night cycle. Mice were acclimated for 7 days before any experimental procedures. The experiments were performed in adherence to the guidelines for the Care and Use of Laboratory Animals of the National Institute of Health.

4.3. Experimental study design

Mice (n = 60) were randomly divided into six groups with 10 mice in each group: (1) control group (0.5% CMC-Na), (2) DSS group, (3) DSS + *Sch B* (*Sch B*, 10 mg/kg), (4) DSS + *Sch B* (*Sch B*, 40 mg/kg), (5) DSS + *Sch B* (*Sch B*, 100 mg/kg). Acute colitis was induced in mice by addition of 2.5% (w/w) DSS in their drinking water for 5 days, followed by 5 d of normal drinking water, while one control group received regular normal drinking water throughout the course of the study. On day 3, all of the animals received saline or drugs intragastrically once a day until the end of the study. At the end of the experiment, mice were euthanized by CO_2 inhalation. Blood collection and colon sampling were done in all groups.

4.4. Histopathological analysis

The colon was excised, rinsed with saline solution, fixed overnight in 4% fresh paraformaldehyde and embedded in paraffin. Sections (5 μm) were obtained, stained with hematoxylin-eosin and observed for pathological changes in the colon tissues under a light microscope. Microscopic sections were graded by the number and severity of lesions.

4.5. Cytokines TNF-α, IL-1β, IFN-γ and IL-6 measurements in colon homogenates

Concentration of TNF-α, IL-1β, IFN-γ and IL-6 in colon tissue homogenates were measured. All the operations were followed by the instruction of the enzymelinked immunosorbent assay (ELISA) kits.

4.6. RT–PCR assay for TNF-α, IL-1β, IFN-γ and IL-6 mRNA expression

Total RNA from colon samples was extracted using the Trizol reagent, according to the manufacturer's instructions. The gene expression levels of TNF-α, IL-1β, IFN-γ and IL-6 in the colon tissue were determined by real-time quantitative reverse-transcription polymerase chain reaction with the use of a StepOnePlusTM system (Applied Biosystems, CA, United States) and specific primers as reported previously. Specific PCR primer pairs for the target genes were: TNF-α, forward 5'-TGAGGTCAATCTGCCCAAGTA-3' and reverse 5'-CAG GGA AGA ATC TGG AAA GGT-3';IL-1β, forward 5'-TTG TGG CTG TGG AGA AG-3' and reverse 5'-ATC AGA GGC AAG GAG G-3'; IFN-γ, forward 5'- AGC GGC TGAC TGA ACT CAG ATT GTA G-3' and reverse 5'- GTC ACA GTT TTC AGC TGT ATA GGG -3'; IL-6, forward 5'-GGA CTG ATG CTG GTG AC-3' and reverse 5'-AGG TTTG CCG AGT AGA T-3'; β-actin, forward 5'-TGG AAT CCT GTG GCA TCC ATG AAAC-3' and reverse 5'-TAA AAC GCA GCT CAG TAA CAG TCC G-3'.

4.7. Western blot analysis for levels of activation of NF-κB and MAPKs signal pathways

Colon tissues were homogenized, washed with PBS, and incubated in lysis buffer in addition to a protease inhibitor cocktail (Sigma, St. Louis, MO) to obtain extracts of colon proteins. Protein concentration was determined by using the Pierce bicinchoninic acid protein assay kit. The equal amounts of proteins (50 mg) were loaded to 10% SDS-PAGE gels and were electro-transferred to nitrocellulose, and then, the proteins were transferred onto the polyvinylidenedifluoride membrane followed by blocking with fat-free milk in Tris-buffered saline (TBS) containing 0.05% Tween-20 followed by incubation with a monoclonal primary polyclonal antibody at 4 °C overnight. Then the membranes were incubated with a 1:5000 dilution (v/v) of secondary antibody after washing three times in TBST. Membranes were washed in TBST three times and antibody binding was visualized with an ECL chemiluminescence system.

4.8. Statistical analysis

The results were presented as mean ± SEM. All data were statistically analyzed with SPSS 11.0 statistical package for Windows version. The means among groups were compared using one-way ANOVA, followed by Student–Newman–Keuls's *post hoc* test. Statistical significance was set at $P < 0.05$.

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