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Anticolitic Effect of the Rhizome Mixture of *Anemarrhena asphodeloides* and *Coptidis chinensis* (AC-mix) in Mice

Se-Eun Jang[1,2], Jin-Ju Jeong[1], Supriya R. Hyam[1], Myung Joo Han[2] and Dong-Hyun Kim[1,*]

[1]*Department of Life and Nanopharmaceutical Sciences and Department of Pharmaceutical Science,*
[2]*Department of Food and Nutrition, Kyung Hee University, Seoul 130-701, Republic of Korea*

Abstract

During a screening program to search the anticolitic herbal medicines, 80% ethanol extract of the rhizome of *Anemarrhena asphodeloides* (AA) was found to potently inhibit the expression of proinflammatory cytokines TNF-α and IL-1β, as well as the activation of NF-κB in LPS-stimulated colonic macrophages, followed by that of the rhizome of *C. chinensis* (CC). AA also potently inhibited TNBS-induced colitic markers, shortening of the colon and increase of macroscopic score, myeloperoxidase activity, TNF-α, IL-1β, and IL-6, in mice. The synergistic effect of CC against the anticolitic effect of AA was investigated. CC synergistically inhibited the anticolitic effect of AA. AC-mix (AA+CC, 1:1) potently inhibited them. AC-mix also inhibited the activation of NF-κB, as well as the expression of TNF-α, IL-1β, IL-6, iNOS and COX-2. The effects of AC-mix against oxazolone-induced colitis were investigated in mice. AC-mix also potently inhibited oxazolone-induced inflammatory markers, colon shortening, macroscopic score, myeloperoxidase activity, NF-κB activation and proinflammatory cytokines. Overall, the anti-colitic effect of AC-mix was superior to that of mesalazine. Based on these findings, AC-mix may improve colitis by inhibiting NF-κB activation.

Key Words: *Anemarrhena asphodeloides*, *Coptidis chinensis*, AC-mix, Colitis

INTRODUCTION

Inflammatory bowel disease (IBS) refers to two chronic relapsing diseases, ulcerative colitis and Crohn's disease, that cause inflammation of the intestines (Shanahan, 2002; Binder, 2004). Their pathogenic mechanism is assumed to be an immune response against gut constituents, such as intestinal bacterial toxins, exposed in intestinal environment (Rafii *et al.*, 1999; Atreya *et al.*, 2000). Among these toxins, bacterial endotoxins, such as lipopolysaccharide (LPS) and flagellin, that can cause colonic inflammation stimulate the mucosal immune system (Radema *et al.*, 1991; Rafii *et al.*, 1999). This induces inflammatory mediators such as pro-inflammatory cytokines, activating the inflammatory reaction via distinct signaling pathways through Toll-like receptors (TLRs) and/or cytokine receptors (Jung *et al.*, 1995; Cario and Podolsky, 2000). Therefore, to cure inflammatory diseases, natural products regulating inflammatory mediators have been attempted (Paradkar *et al.*, 2004; Joh *et al.*, 2011).

The rhizome of *Anemarrhena asphodeloides* (family Liliaceae) is an antipyretic, antiphlogistic, sedative, and diuretic agent in traditional Chinese medicine. Its main constituents are saponins. Of these saponins, timosaponins improve learning and memory in rats (Hu *et al.*, 2005; Li *et al.*, 2007) and inhibit platelet aggregation factor-induced platelet aggregation (Dong and Han, 1991). Xanthones, such as mangiferin, inhibit lung inflammation and pruritus in mice (Lee *et al.*, 2009; Rivera *et al.*, 2011), inhibited LPS-induced iNOS and COX-2 expression in RAW264.7 cells (Shin *et al.*, 2008) and protect scopolamine-induced brain impairment in mice (Jung *et al.*, 2009). However, the anti-colitic effect of AA remains to be thoroughly studied.

The rhizome of *Coptidis japonica* (family Ranunculaceae), whose main constituent is an isoquinolone alkaloid berberine, has been used to treat patients who have gastroenteritis and diarrhea in tradition Chinese medicine (Bae *et al.*, 1998; Zhou and Mineshita, 2000). Although its closely related plants are *C. chinensis*, *C. teeta*, and *C. sinensis*, their constituents are different to that of *C. japonica*. Nevertheless, they contain berberine. Berberine was proved to have biological activities

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***Corresponding Author**
E-mail: dhkim@khu.ac.kr
Tel: +82-2-961-0374, Fax: +82-2-957-5030

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such as bacteriocidal, anticholera toxin, cholesterol-lowering, anti-inflammatory, and anticolitic effects (Zhou and Mineshita, 2000; Cernáková and Kostálová, 2002; Kong *et al.*, 2004; Yu *et al.*, 2005; Lee *et al.*, 2010). Berberine also inhibited IL-8 production in colonic epithelial cells and improved TNBS-induced colitis in mice (Zhou and Mineshita, 2000). In our previous study, berberine ameliorated TNBS-induced colitis in mice by inhibiting lipid peroxidation and NF-κB activation (Lee *et al.*, 2010).

During an herbal medicine screening program to search anticolitic agents, 80% ethanol extract of *Anemarrhena asphodeloides* (AA) potently inhibited the inflammation in LPS-stimulated colonic macrophages and 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice. Therefore, to develop the potent anticolitic agent, we evaluated anti-colitic effects of some herbal medicines, which also exhibited anti-colitic effect, in the presence of AA in TNBS- or oxazolone-induced colitis in mice.

MATERIALS AND METHODS

Materials

RPMI1640, TNBS, oxazolone, hexadecyl trimethyl ammonium bromide, and radio-immunoprecipitation assay (RIPA) lysis buffer were purchased from Sigma (St Louis, MO, USA). The protease inhibitor cocktail was purchased from Roche Applied Science (Mannheim, Germany). Enzyme-linked immunosorbent assay (ELISA) kits were from Pierce Biotechnology, Inc., (Rockford, IL, USA). Antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA) and Cell Signaling (Cell Signaling Technology, Inc. (Danvers, MA, USA). The enhanced chemiluminescence (ECL) immunoblot system was from Pierce Co. (Rockford, IL, USA).

Preparation of herbal medicine extracts

The rhizomes of *Anemarrhena asphodeloides* (AA) and *C. chinensis* were purchased from Kyung-Dong Market, Seoul, Korea, and were taxonomically identified by Prof. Nam-Jae Kim, an adjunct professor of Kyung Hee University (Seoul, Korea). Voucher specimens (KHUP1203051-1203052) were deposited at the Herbarium of Kyung Hee University, Seoul, Korea. Each pulverized herbal medicine (100 g) was extracted three times with 80% EtOH under water bath. Their 80% EtOH extracts (named AA and CC, respectively) were combined and evaporated to dryness under reduced pressure, which yielded 33 g and 16 g, respectively. AC-mix was the mixture of AA and CC (=1:1). The content of mangiferin and berberine in AC- mix were 0.31% and 2.02%, respectively.

Animals

Male C57BL/6 (18-22 g, 6 weeks) were supplied from the Orient Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 20-22°C and 50 ± 10% humidity, fed standard laboratory chow (Samyang Co., Seoul, Korea), and allowed water ad libitum. All procedures relating to animals and their care conformed to the international guidelines 'Principles of Laboratory Animals Care' (NIH publication no. 85-23 revised 1985 and Kyung Hee University, animal experiment guideline 2006).

Isolation and culture of intestinal macrophages

For the isolation of macrophages in intestine, the Payer's patches were isolated from the mouse intestines and were digested in DMEM containing 1 mg/ml dispase, 0.25 mg/ml collagenase A, and 25 U/ml DNAse (Roche Diagnostic, Indianapolis, IN, USA) at 37°C for 20 min with shaking and passed through a 70 μm cell strainer (BD Biosciences, San Diego, CA, USA). The cells were harvested by centrifugation and washed with PBS containing 4% FBS. The cells were resuspended in cold IMag buffer (0.05% bovine serum albumin and 2 mM EDTA in PBS) and viable cells were counted with trypan blue. The colonic macrophages were isolated from colonic cells by using a biotin-labeled anti-mouse F4/80 antibody and streptoviridin magnetic beads (Invitrogen).

To examine the anti-inflammatory effect of test agents, intestinal macrophages were incubated in the absence or presence of test agents with 50 ng/ml LPS. The cytoxicities of these agents in the cell viability were measured using the crystal violet method.

Preparation of TNBS-induced colitic mice

TNBS-induced colitis was induced by the administration of 2.5% (w/v) TNBS solution (100 μl, in 50% ethanol) into the colon of lightly anesthetized mice via a thin round-tip needle equipped with a 1-ml syringe (Joh *et al.*, 2011). The normal group was treated with vehicle alone. The needle was inserted so that the tip was 3.5-4 cm proximal to the anal verge. To distribute the agent within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >95% of the mice retained the TNBS enema. If the mice quickly excreted the TNBS-ethanol solution, it was excluded from the remainder of the study. Test agents (dissolved in 2% tween 80 solution, 10 or 20 mg/kg) or vehicle alone was orally administered once a day for 3 days after TNBS treatment. The mice were sacrificed the next day after the final administration of test agents. The colon was quickly removed, opened longitudinally, and gently cleared of stool by PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulceration and no inflammation; 1, ulceration without hyperemia; 2, no ulceration and local hyperemia; 3, ulceration and inflammation at one site only; 4, two or more sites of ulceration and inflammation; 5, ulceration extending more than 2 cm), and the colon tissue was then used for ELISA and immunoblotting (Joh *et al.*, 2011).

Preparation of oxazolone-induced colitis in mice

Oxazolone-induced colitis was studied in mice as previously described (Wirtz *et al.*, 2007). To presensitize the mice, a 1.5 cm×1.5 cm field was shaved on the back between the shoulders, and 200 μl of a 3% (w/v) solution of oxazolone in acetone/olive oil (4:1) was applied. Eight days after presensitization, mice were rechallenged intrarectally under ethyl ether general anesthesia with 100 μl of 1% oxazolone in 50% ethanol to induce colitis or 50% ethanol alone (vehicle control). Test agents (AA, 10 and 20 mg/kg; AC, 10 and 20 mg/kg; mesalazine, 10 mg/kg dissolved in 2% tween 80) were orally administered once a day for 15 days after oxazolone administration. The mice were sacrificed on next day after the final administration of test agents. The colon was quickly removed, opened longitudinally, and gently cleared of stool by PBS. Macroscopic assessment of the disease grade was

scored according to a previously reported scoring system and the colon tissue was then used for ELISA and immunoblotting (Joh *et al.*, 2011).

Histological exam

The colons were fixed in 10%-buffered formalin solution, embedded in paraffin using standard methods, cut into 8-μm sections, stained with hematoxylin-eosin, and then assessed under light microscopy (Joh *et al.*, 2011).

Colon tissue preparation

The colon tissues were excised, perfused with ice-cold perfusion solution (0.15 M KCl, 2 mM EDTA, pH 7.4), and homogenized in 50 mM Tris-HCl buffer (pH 7.4) (Joh and Kim, 2011). The homogenates were centrifuged at 10,000×g at 4°C for 30 min and the supernatants used for further studies.

Assay of myeloperoxidase activity

An aliquot (50 μl) of the colon supernatant was added to a reaction mixture of 1.6 mM tetramethyl benzidine and 0.1 mM H_2O_2 and incubated at 37°C; the absorbance was obtained at 650 nm over time (Joh *et al.*, 2011). Myeloperoxidase activity was defined as the quantity of enzyme degrading 1 μmol/ml of peroxide at 37°C and expressed in unit/mg protein. The protein content was assayed by the method of Bradford (1976).

ELISA and immunoblotting

For the ELISA of IL-1β, IL-6, IL-10 and TNF-α, colons were homogenized in 1 ml of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. The lysate or cell-cultured supernatants was centrifuged (15,000×g, 4°C) for 15 min, and the supernatant was transferred to 96-well ELISA plates. IL-1β, IL-6, IL-10 and TNF-α concentrations were determined using commercial ELISA kits (Pierce Biotechnology, Inc., Rockford, IL, USA) (Joh *et al.*, 2011).

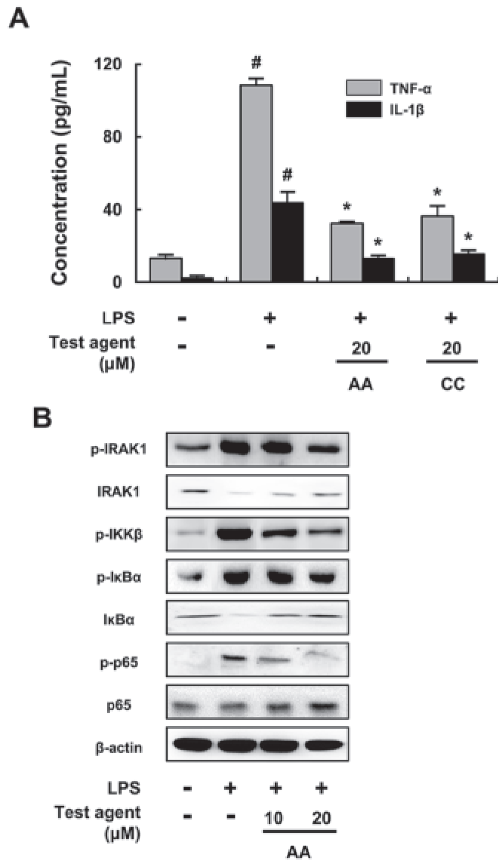

Fig. 1. Anti-inflammatory effect of 80% ethanol extracts of Anemarrhenae Rhizoma (AA) and Coptidis rhizoma (CC) in LPS-stimulated intestinal macrophages. (A) Effects of AA and CC on the expression of TNF-α and IL-1β (A). (B) Effect of AA on IRAK1, IKKβ and IκBα phosphorylation and NF-κB activation. Colonic macrophages ($0.5×10^6$ cells) were treated with 50 ng/ml LPS in the absence or presence of test agents (10 and 20 μM) for 20 h. The normal control group was treated with vehicle alone. The levels of these cytokines in culture supernatants were measured by ELISA and p65, p-p65, IRAK1, p-IRAK1, p-IKKβ, IκBα, and p-IκBα were measured by immunoblotting. *Significantly different vs. group treated with LPS alone (p<0.05), #Significantly different vs. LPS-non-treated group (p<0.05).

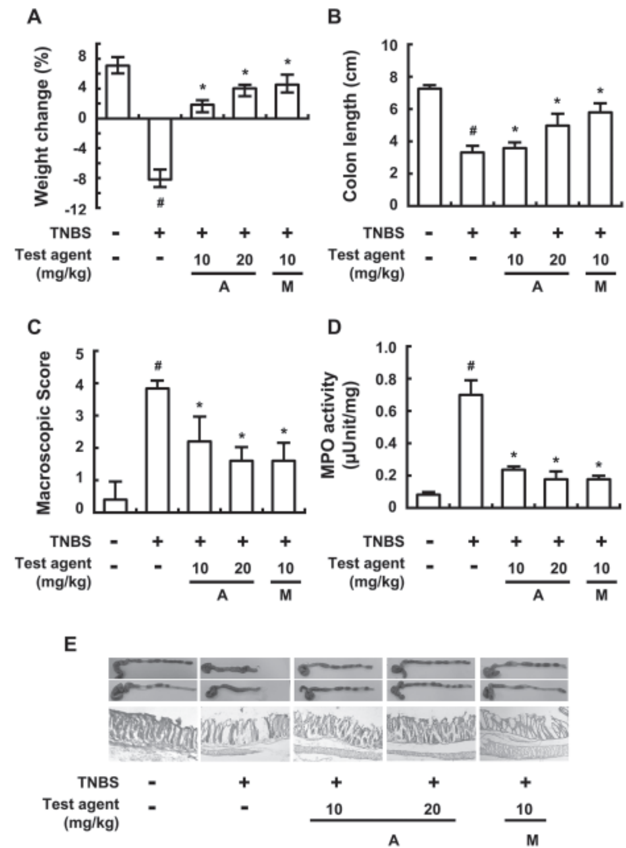

Fig. 2. Inhibitory effect of 80% ethanol extract of Anemarrhenae Rhizoma on body weight (A), colon length (C), macroscopic score (B), myeloperoxidase (MPO) (D), myeloperoxidase (MPO) and histology (E) in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline or test agents. Test compounds [80% ethanol extract of Anemarrhenae Rhizoma (A, 10 and 20 mg/kg), mesalazine (M; 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. All values are the mean ± S.D. (n=6). #p<0.05 vs. normal control group, *p<0.05 vs. TNBS group.

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For the immunoblot analyses of p-IRAK1, p-IKKβ, p-p65 and β-actin, the colon tissue homogenates or the colleted cells were resuspended in 1 ml of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail (Joh *et al.*, 2011). After centrifugation, the supernatant was used for the immunoblot assay. The proteins from collected cells were subjected to electrophoresis on 8-10% sodium dodecyl sulfate-polyacrylamide gel and then transferred to nitrocellulose membrane. Levels of p-IRAK1, p-IKKβ, p-p65 and β-actin were assayed as previously described. Immunodetection was performed using an enhanced chemiluminescence detection kit.

Statistical analysis

All data are expressed as the mean ± standard deviation (S.D.), with statistical significance analyzed using one-way ANOVA followed by a Student-Newman-Keuls test.

RESULTS

During a screening program to search the anticolitic herbal medicines, 80% EtOH extract of AA was found to potently inhibit the expression of proinflammatory cytokines TNF-α and IL-1β in LPS-stimulated intestinal macrophages, followed by CC (Fig. 1). AA also inhibited the phosphorylation, of IRAK1,

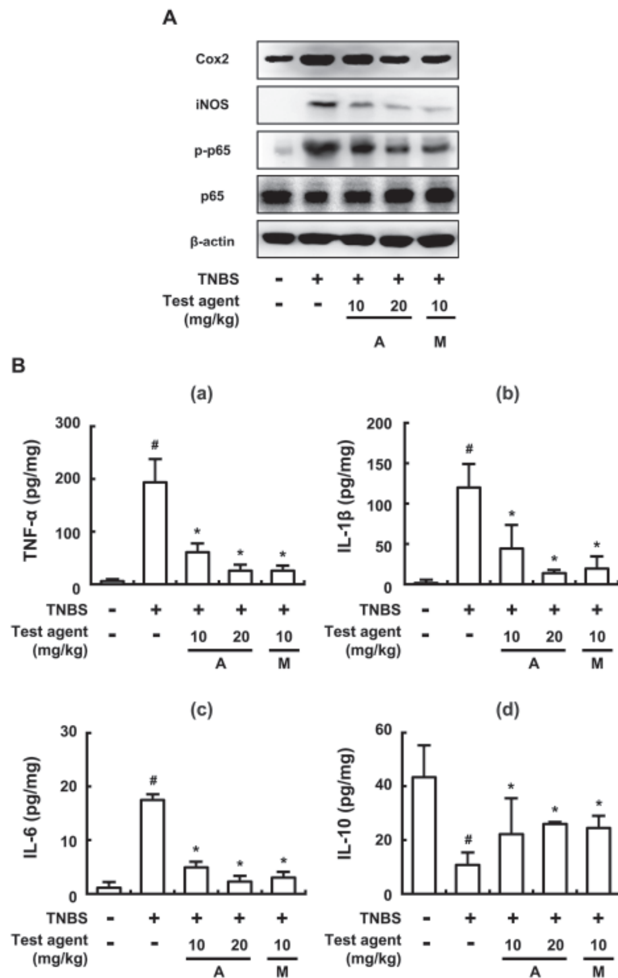

Fig. 3. Inhibitory effect of 80% ethanol extract of Anemarrhenae Rhizoma on the activation of NF-κB and the expression of COX-2, iNOS, TNF-α, IL-1β, IL-6 and IL-10 in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline or test agents. Test compounds [80% ethanol extract of Anemarrhenae Rhizoma (A, 10 or 20 mg/kg), mesalazine (M, 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. (A) NF-κB, IRAK1, COX-2, iNOS, and β-actin were analyzed by immunoblotting. (B) TNF-α (a), IL-1β (b), IL-6 (c), IL-10 (d) were analyzed by ELISA kits. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, *p<0.05 vs. TNBS group.

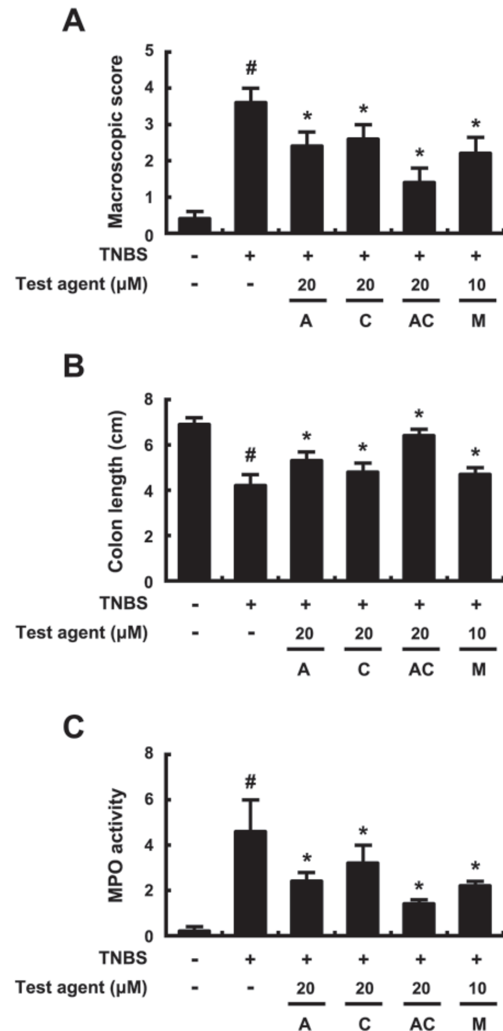

Fig. 4. Inhibitory effect of 80% ethanol extracts of Anemarrhenae Rhizoma (AA) and Coptidis rhizoma (CC) and AC-mix on colon length (A), macroscopic score (B) and myeloperoxidase (MPO) (C) in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, AA, CC, AC mix or mesalazine. Test compounds [AA (A), CC (C) and AC-mix (20 mg/kg), mesalazine (M, 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, *p<0.05 vs. TNBS group.

IKKβ and IκBα and the activation of NF-κB.

The ability of AA to inhibit TNBS-induced colitis was tested in mice (Fig. 2). Intrarectal injection of TNBS significantly caused colitis, manifested by shortening of the colon and increase of myeloperoxidase activity. AA also potently inhibited TNBS-induced shortening of colons and increase of myeloperoxidase activity. AA also inhibited TNBS-induced NF-κB activation, as well as expression of proinflammatory cytokines, TNF-α, IL-1β, and IL-6 (Fig. 3).

Next, we tested the synergistic effect of CC in the anticolitic effect of AA in mice (Fig. 4). Intrarectal injection of TNBS potently caused severe inflammation, manifested by colon shortening and myeloperoxidase increasing. Treatment with AA or CC in TNBS-induced colitic mice significantly inhibited the shortening of colons and the increase of myeloperoxidase activity. Treatment with AC-mix prepared by the addition of CC in AA inhibited the shortening of colons and the increase of myeloperoxidase activity more potently than those of AA or CC.

Next, we tested the anticolitic effect of AC in mice (Fig. 5). Intrarectal injection of TNBS caused severe inflammation, manifested by shortened, thickened, and erythematous colons. TNBS also increased myeloperoxidase activity. Treatment with the combined mixture AC-mix in TNBS-induced colitic mice inhibited the shortening of colons and the increase of myeloperoxidase activity. AC-mix also inhibited NF-κB activation (Fig. 6). AC-mix at a dose of 20 mg/kg inhibited TNBS-induced TNF-α by 74.1%, IL-1β by 81.6%, and IL-6 expression by 82.8%. AC-mix also inhibited iNOS and COX-2 expression. However, it reversed IL-10 to 71.9% of the normal control group. The anti-colitic effect of AC-mix at a dose of 20 mg/kg was more potent than at a dose of 10 mg/kg. However, in our preliminary study treated with 10 or 50 mg/kg mesalazine to TNBS-induced colitic mice, we could not find the significant differences of anti-colitic effects, such as colon shortening and macroscopic score, between them (data not shown). Thus, the anti-colitic effect of AC-mix was superior to that of mesalazine.

Next, we investigated the effect of AC-mix against oxazolone-induced colitis in mice (Fig. 7). Treatment with oxazolone alone caused severe colonic inflammation, including the

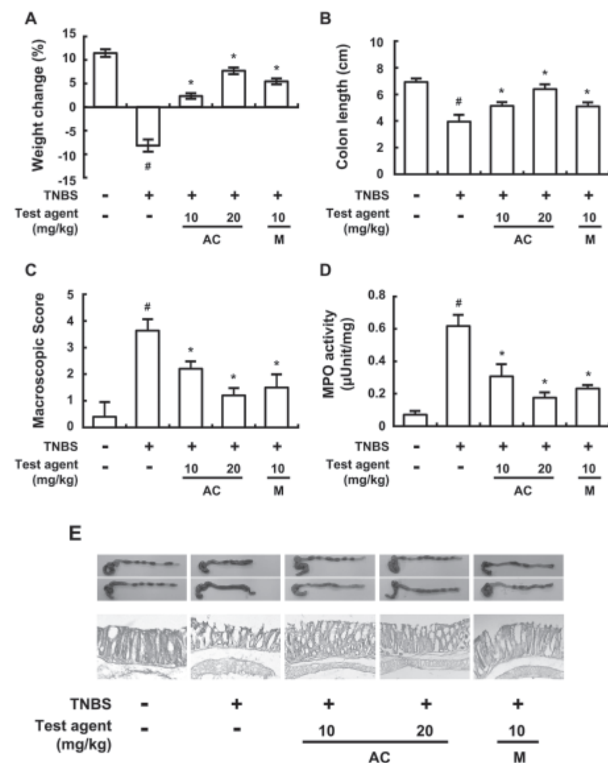

Fig. 5. Inhibitory effect of AC-mix on body weight (A), colon length (C), macroscopic score (B), myeloperoxidase (MPO) (D), and histology (E) in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, AC mix or mesalazine. Test compounds [AC-mix (AC, 10 and 20 mg/kg, mesalazine (M, 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, $^{*}p$<0.05 vs. TNBS group.

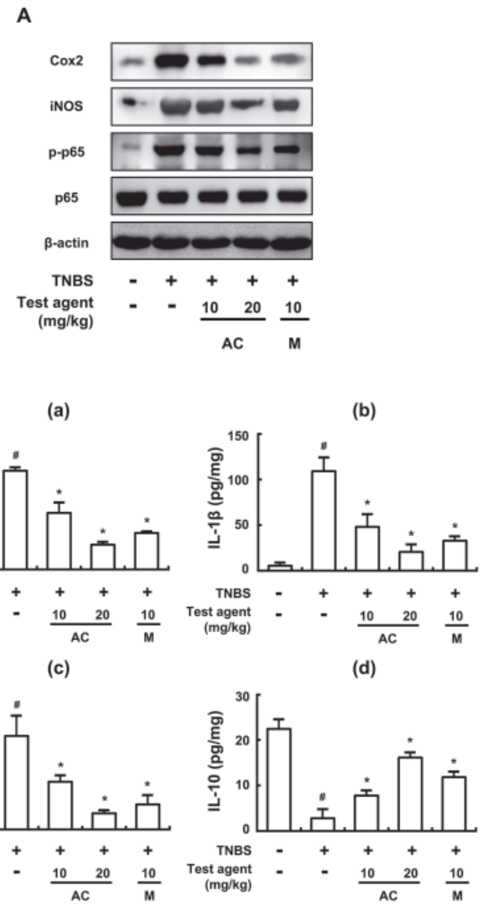

Fig. 6. Inhibitory effect of AC-mix on the activation of NF-κB and the expression of COX-2, iNOS, TNF-α, IL-1β, IL-6 and IL-10 in TNBS-induced colitic mice. TNBS, except in the control group, was intrarectally administered to mice treated with saline, AC mixture or mesalazine. Test compounds [AC-mix (10 or 20 mg/kg), mesalazine (MS; 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. (A) NF-κB, COX-2, iNOS, and β-actin were analyzed by immunoblotting. (B) TNF-α (a), IL-1β (b), IL-6 (c), IL-10 (d) were analyzed by ELISA kits. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, $^{*}p$<0.05 vs. TNBS group.

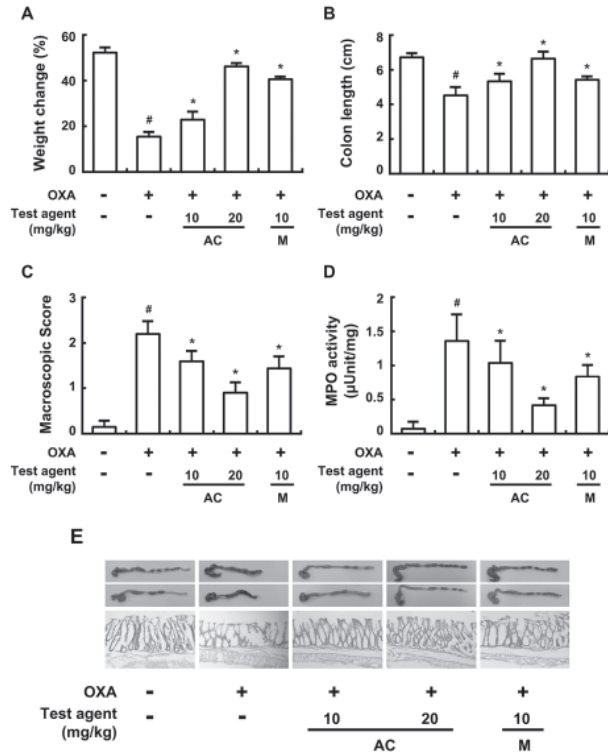

Fig. 7. Inhibitory effect of AC-mix on body weight (A), colon length (C), macroscopic score (B), myeloperoxidase (MPO) (D), myeloperoxidase (MPO) and histology (E) in oxazolone-induced colitic mice. Oxazolone, except in the control group, was intrarectally administered to mice treated with saline, AC mixture or mesalazine. Test compounds [AC-mix, (AC, 10 or 20 mg/kg), mesalazine (M,10 mg/kg), or saline] were orally administered for 3 days after oxazolone treatment. The mice were sacrificed at 20 h after the final administration of test agents. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, *p<0.05 vs. oxazolone group.

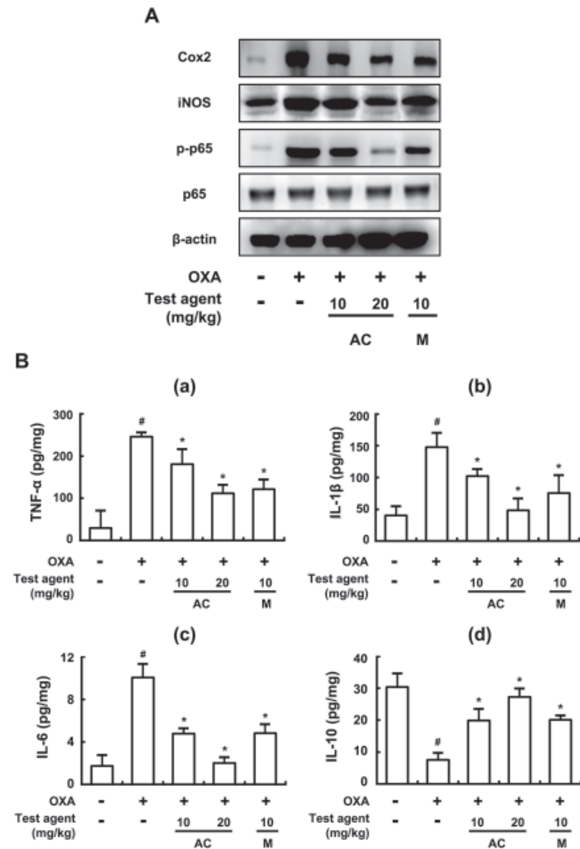

Fig. 8. Inhibitory effect of AC-mix on the activation of NF-κB and IRAK1 and the expression of COX-2, iNOS, TNF-α, IL-1β, IL-6 and IL-10 in oxazolone-induced colitic mice. Oxazolone, except in normal control group, was intrarectally administered to mice treated with saline, AC mixture or mesalazine. Test compounds [AC mixure (AC, 10 or 20 mg/kg), mesalazine (M, 10 mg/kg), or saline] were orally administered for 3 days after TNBS treatment. The mice were sacrificed at 20 h after the final administration of test agents. (A) NF-κB, COX-2, iNOS, and β-actin were analyzed by immunoblotting. (B) TNF-α (a), IL-1β (b), IL-6 (c), IL-10 (d) were analyzed by ELISA kits. All values are the mean ± S.D. (n=6). $^{\#}p$<0.05 vs. normal control group, *p<0.05 vs. oxazolone group.

shortening of colon, loosing of bodyweight, and an increase of macroscopic score, myeloperoxidase activity, proinflammatory cytokine expression, and NF-κB activation. AC-mix potently ameliorated oxazolone-induced colon shortening and bodyweight loosing, as well as increased myeloperoxidase activity, proinflammatory cytokine expression and NF-κB activation. AC-mix also inhibited iNOS and COX-2 expression (Fig. 8). AC-mix at a dose of 20 mg/kg inhibited TNBS-induced TNF-α by 54.6%, IL-1β by 67.3%, and IL-6 expression by 80.0%. However, AC-mix reversed IL-10 to 89.7% of the normal control group. AC-mix also inhibited TNBS-induced IRAK1 and IKKβ phosphorylation, as well as NF-κB activation. Overall, the anti-colitic effect of AC-mix was superior to that of mesalazine.

DISCUSSION

Acute and chronic inflammations are the body's response to injury or infection (Shanahan, 2002; Binder, 2004). Acute inflammation is a normal and helpful response to injury. However, chronic inflammation is persistent and excessive. This inflammatory response causes progressive damage to the body, leading to a variety of diseases, such as colitis, rheumatoid arthritis and even cancer. The inflammatory reactions can be mediated by inflammatory mediators, including IL-1β, IL-6, TNF-α, IFN-γ, nitric oxide and prostaglandins in immune cells (Medzhitov and Janeway, 2000; Berrebi *et al.*, 2003). Of these inflammatory mediators, pro-inflammatory cytokines such as IL-1β and TNF-α are activated through NF-κB. Bacterial lipopolysaccharide (LPS) increases blood IL-1β and TNF-α levels via TLR4, leading to inflammation, although blood IL-1β and TNF-α level are barely detectable in mice without any stimuli or treatment (Ingalls *et al.*, 1999; Aderem and Ulevitch, 2000). TLR4 recognizes LPS on the membrane of immune cells, and initiates a signaling cascade through the Toll/IL-1R (TIR) domain of its cytoplasmic tail and MyD88, allowing for subsequent activation of IL-1R-associated kinases (IRAKs) (Cario and Podolsky, 2000; O'Neill and Dinarello, 2000). All IRAK members form multimeric receptor complexes. Phosphory-

lated IRAK1 activates a multimeric protein complex, leading to NF-κB activation and proinflammatory cytokine expression.

In the present study, AA potently ameliorated TNBS-induced colitis in mice. Furthermore, AA inhibited inflammation in LPS-stimulated colonic macrophages by regulating the phosphorylation of IRAK1 and the activation of NF-κB. AC-mix, which was prepared by the addition of CC in AA, ameliorated TNBS- or oxazolone-induced colitis via inhibiting the activation of NF-κB in the colons of mice. Thus, the anticolitic effect of AA was synergistically increased by the addition of CC. Oeverall, the anticolitic effect of AC-mix was superior to that of mesalazine, which is used commercially for colitis.

Based on these findings, AC-mix may potently ameliorate colitis by synergistically inhibiting NF-κB signaling pathway.

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