Oral administration of fermented wild ginseng ameliorates DSS-induced acute colitis by inhibiting NF-κB signaling and protects intestinal epithelial barrier

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Ginseng has been widely used for therapeutic and preventive purposes for thousands of years. However, orally administered ginseng has very low bioavailability and absorption in the intestine. Therefore, fermented ginseng was developed to enhance the beneficial effects of ginseng in the intestine. In this study, we investigated the molecular mechanisms underlying the anti-inflammatory activity of fermented wild ginseng (FWG). We found that FWG significantly alleviated the severity of colitis in a dextran sodium sulfate (DSS)-induced colitis mouse model, and decreased expression level of pro-inflammatory cytokines in colonic tissue. Moreover, we observed that FWG suppressed the infiltration of macrophages in DSS-induced colitis. FWG also attenuated the transcriptional activity of nuclear factor-κB (NF-κB) by reducing the translocation of NF-κB into the nucleus. Our data indicate that FWG contains anti-inflammatory activity *via* NF-κB inactivation and could be useful for treating colitis. [BMB Reports 2015; 48(7): 419-425]

INTRODUCTION

Inflammatory bowel disease (IBD) is a chronic inflammatory disease of the gastrointestinal tract such as Crohn's disease and ulcerative colitis (1). The number of patients with IBD is rising in developed countries, and IBD is a public health problem

worldwide (2). The existing therapy for IBD often has marginal results, with a high likelihood of additional side effects. Therefore, it is important to develop a new effective and safe therapy for IBD. Recently, herbal medicines/supplements are being used as alternative treatment for IBD (3). Although the cause of IBD is not fully known, several lines of evidence suggest that IBD is due to mucosal barrier dysfunctions, leading to improper immune responses to intestinal microflora, modifications in the composition of microflora, or alterations in tight junction protein expression. These damaged barrier functions are due to high expression of pro-inflammatory cytokines, such as TNF-α and IL-1β (4).

Dextran sodium sulfate (DSS)-induced colitis model is generally a good model of experimental colitis because it has similar symptoms as human IBD (5). Histologically, the DSS-induced colitis model is defined by crypt damage, epithelial ulceration and mucosal edema in association with the infiltration of inflammatory cells such as macrophages (6, 7). These processes allow the entry of bacteria into the mucosal layer, which causes the activation of intestinal macrophages (8, 9).

Panax ginseng C. A. Meyer (Korea or Asian ginseng) has been widely used as an herbal medicine for centuries in East Asia. The pharmacological effects of ginseng have been confirmed in cancer (10), obesity (11), cardiovascular diseases (12) and inflammation (13). Ginsenosides, metabolites of ginseng, are known as the major active compounds, with a number of pharmacological activities including anti-cancer and anti-inflammatory properties (14). Fermented ginseng has been developed to improve the beneficial effects of ginseng with increased amounts of various ginsenosides such as Rh1, and Compound K (C-K) (15, 16). Although many comparative studies have been conducted on the therapeutic aspects of ginseng and fermented ginseng, there are no reports on the effects of fermented wild ginseng (FWG) on IBD.

Therefore, we investigated the anti-inflammatory effects of FWG on a DSS-induced colitis mouse model by examining histological changes and inflammatory responses such as the production of pro-inflammatory cytokines and the infiltration of

macrophages. Furthermore, we used LPS-induced RAW264.7 and mouse peritoneal macrophages to explore the molecular mechanism of FWG in inflammatory conditions.

RESULTS

Changes in ginsenosides composition from fermented wild ginseng

Many studies have reported that the conversion of major ginsenosides in ginseng into minor metabolites through the fermentation process improved bioactivity and bioavailability. To investigate whether our fermentation process increases the amount of minor metabolites, we analyzed the minor ginsenoside metabolite contents of wild ginseng (WG) and FWG using HPLC. During fermentation, the contents of C-K, 20(S)-protopanaxatriol (PPT), Rh1, F1 and 20(S)-protopanaxadiol (PPD), which are ginsenosides absent in WG, increased drastically in FWG from 0 mg/g to 3.32, 1.16, 0.89, 1.5, and 1.3 mg/g, respectively (Fig. 1A). To investigate whether the increased amount of minor ginsenosides in FWG have a greater suppressive effect on the inflammatory response than WG, we investigated p65 phosphorylation levels in WG and FWG treatment. We found that FWG has a significant inhibitory effect against LPS-induced inflammatory responses in RAW264.7 compared with WG. The results show that FWG contain more active ginsenosides than non-fermented wild ginseng, suggesting that FWG may have more therapeutic effects.
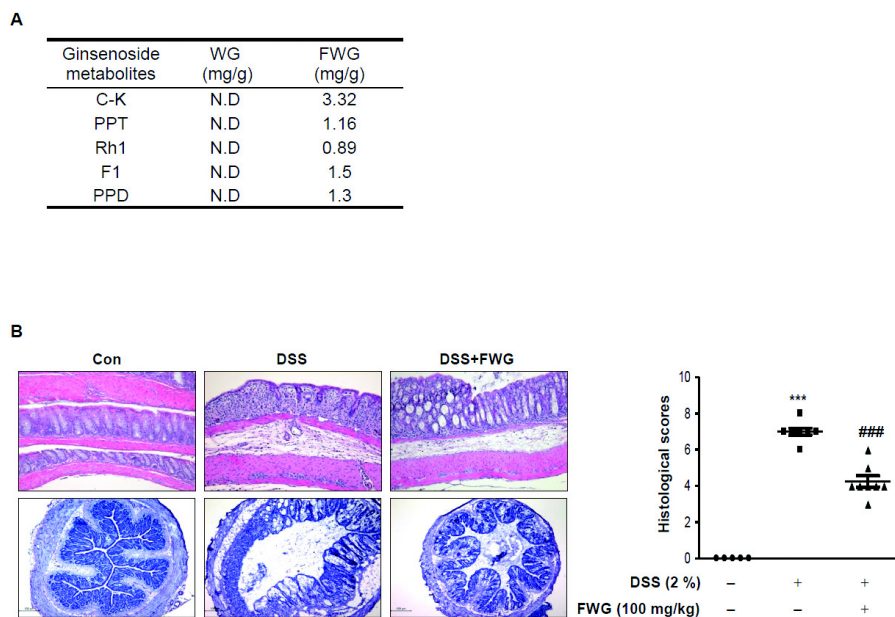
FWG ameliorates DSS-induced colitis in mice

To evaluate the effect of FWG on DSS-induced colitis, mice were pretreated with FWG (100 mg/kg/day, P.O.) for 3 weeks.

The mice were then exposed to DSS (2%) in their drinking water for 7 days with or without FWG. The DSS-treated mice developed acute colitis with severe inflammation, and crypt damage. These changes were reduced by pre-treatment with FWG. The histological score was significantly lower in the FWG-pretreated group (4.25 ± 0.89, P < 0.005) compared with the DSS-treated group (7 ± 0.53) (Fig. 1B). These results show that FWG treatment attenuated the severity of DSS-induced colitis.

FWG inhibits pro-inflammatory responses and prevents loss of ZO-1 in colon of DSS-treated mice

Increase in pro-inflammatory cytokines is a hallmark of inflammation in IBD (17). Therefore, we investigated the effects of FWG on pro-inflammatory cytokine production in the DSS-induced colitis model. Pre-treatment with FWG down-regulated DSS-induced mRNA levels of IL-1β, IL-6, and IL-12p40, TNF-α and IFN-γ, which are pro-inflammatory cytokines in colonic tissues (Fig. 2A). Similarly, we observed that FWG administration led to a decrease in DSS-induced TNF-α protein level in colonic tissue (Fig. 2B). In addition, FWG reduced the number of F4/80$^+$ macrophages in DSS-treated mice compared to mice treated with only DSS. This means that FWG suppressed macrophage infiltration into the colon in DSS-treated mice (Fig. 2C). These data suggest that FWG alleviates DSS-induced colitis by controlling innate immunity, such as by regulating macrophage-produced cytokines including TNF-α.

Several studies reported that IBD was characterized by a reduction in ZO-1, a tight junctional protein. Alteration of tight junction complexes induces dysfunction in the epithelial bar-

A

Ginsenoside metabolites	WG (mg/g)	FWG (mg/g)
C-K	N.D	3.32
PPT	N.D	1.16
Rh1	N.D	0.89
F1	N.D	1.5
PPD	N.D	1.3

B

Fig. 1. Fermented wild ginseng (FWG) ameliorates DSS-induced colitis. (A) Minor ginsenoside metabolite contents of wild ginseng (WG) and FWG (B) Mice were administered 2% DSS in drinking water for 7 days with or without FWG (100 mg/kg). The paraffin sections were stained with hematoxylin and eosin for histological scores. Longitudinal section (upper, magnification, ×200) and cross section (lower, magnification, x100). Values are expressed as mean ± SD, n = 8. ***P < 0.001, versus vehicle-treated mice. ###P < 0.001, versus DSS-treated mice.
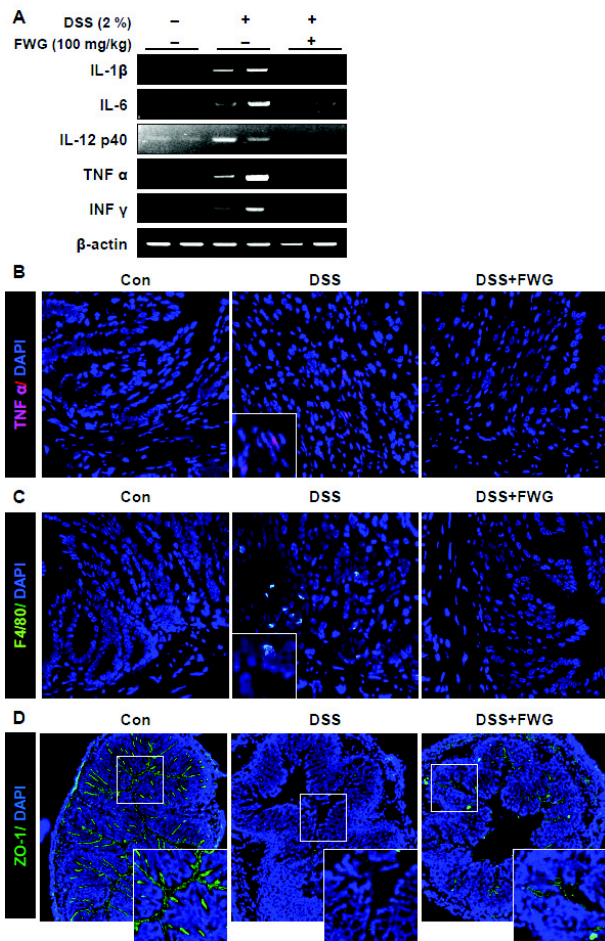
Fig. 2. FWG suppressed production of pro-inflammatory cytokines and recovers lost ZO-1 in colon of DSS-induced colitis mice. (A) RT-PCR analysis was used for the determination of pro-inflammatory cytokine mRNA levels. (B) Immunofluorescence staining of representative frozen sections for the determination of TNF α level (red), (C) F4/80 (green) positive macrophage infiltrations and (D) ZO-1 distribution/expression; control, 2% DSS treated and 2% DSS plus 100 mg/kg FWG treated mice. Nuclei were stained with DAPI (blue); (B, C) Magnification, ×200, (D) Magnification, ×40.

rier (18, 19). To further explore the beneficial effects of FWG on the DSS-induced colitis model, we studied whether pre-treatment with FWG had a protective effect on the intestinal barrier in DSS-mediated colitis. We found that FWG pre-treatment prevented DSS-mediated zonula occludens-1 (ZO-1) reduction from tight junction complexes in colonic epithelial cells (Fig. 2D). The results suggest that FWG is involved in the distribution of tight junction complexes and improve intestinal barrier functions.
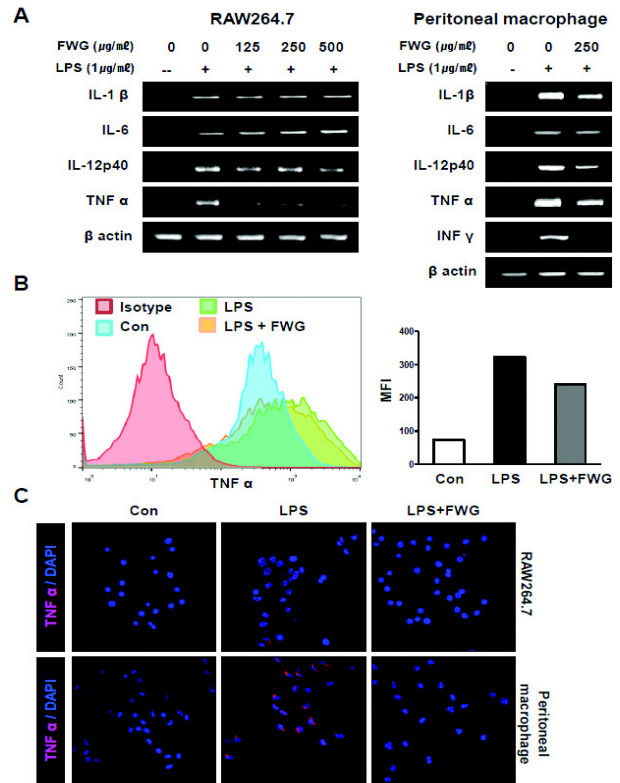
Fig. 3. FWG reduces production of pro-inflammatory cytokines in RAW264.7 macrophages and peritoneal macrophages *in vitro*. The cells were pretreated with FWG for 1 h and then induced with LPS (1 μg/ml) for 4 h. (A) mRNA levels of pro-inflammatory cytokines were determined by RT-PCR. (B) TNF-α level in RAW 264.7 cell line was determined by FACS analysis. Histogram shows mean fluorescence intensity (MFI) of TNF-α$^+$ cells. (C) TNF-α level (red) in RAW264.7 and peritoneal macrophages were determined by immunofluorescence assay. Nuclei were stained with DAPI (blue). Magnification, ×600.

FWG reduces production of pro-inflammatory cytokines in RAW264.7 macrophage and peritoneal macrophage *in vitro*

It was reported that LPS-stimulated production of pro-inflammatory cytokines is caused by NF-κB activation and suppression of NF-κB activity leads to reduction of those cytokines (20). To identify the mechanism involved in the anti-inflammatory response by FWG, we investigated whether FWG inhibits LPS-induced pro-inflammatory cytokine production *in vitro*. FWG decreased LPS-induced up-regulation of TNF-α and IL-12 p40, but not IL-1β and IL-6, at the mRNA levels in a concentration dependent manner in RAW264.7 macrophages. In peritoneal macrophages, pre-treatment with FWG reduced LPS-induced up-regulation of IL-1β, IL-6, IL-12p40, TNF-α, and IFN-γ mRNA levels (Fig. 3A). Similarly, FWG suppressed LPS-induced up-regulation of TNF-α protein level as shown in FACS and immunofluorescence assays in RAW264.7 macrophages
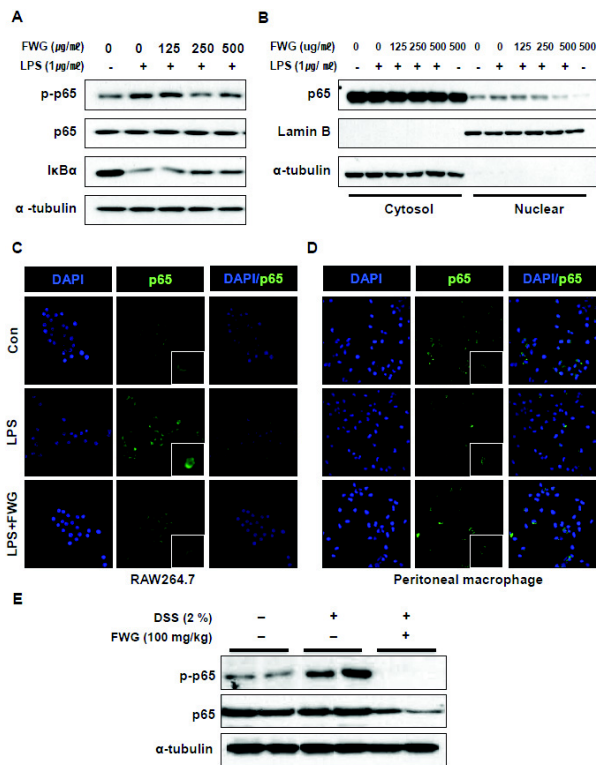
Fig. 4. FWG inhibits the NF-κB activation in inflammatory conditions *in vitro* and *in vivo*. (A) RAW264.7 macrophage cell lines were pretreated with FWG (125, 250, 500 μg/ml) for 1h and then stimulated with LPS (1 μg/ml) for 30 min. Western blot analysis for IκB-α, p-p65, and p65 are shown. (B) RAW264.7 macrophage cell lines were pretreated with FWG (125, 250, 500 μg/ml) for 1 h and stimulated with LPS (1 μg/ml) for 1 h. Nuclear and cytosolic extracts were isolated and the levels of p65 in each fraction were determined by western blot. Lamin B and α-tubulin were used as internal controls. (C) RAW264.7 macrophage cell line and (D) peritoneal macrophage were pretreated with 250 μg/ml FWG for 1 h and then stimulated with LPS (1 μg/ml) for 1 h. NF-κB nuclear translocation was detected by immunofluorescence assay using an antibody for p65 (green). Nuclei were stained with DAPI (blue). Magnification, ×600. (E) Total proteins were prepared from colon tissues of control, 2% DSS treated and 2% DSS plus 100 mg/kg FWG treated mice. The levels of p-p65 and p65 were determined by western blot.

and peritoneal macrophages from mice (Fig. 3B, C). Consistent with the *in vivo* data, we confirmed that treatment with FWG reduced the expression of pro-inflammatory cytokines *in vitro*.

FWG suppress NF-κB signaling pathway *in vitro* and *in vivo*
The activation of NF-κB is significantly induced in IBD patients (21). Therefore, we investigated whether FWG abrogates NF-κB signaling *in vitro*. We found that pretreatment with FWG inhibited LPS-induced degradation of IκB-α and phosphorylation of NF-κB (Fig. 4A). We also found that FWG significantly suppressed the LPS-induced translocation of the

NF-κB subunit, p65, to the nucleus in RAW264.7 (Fig. 4B, C) and peritoneal macrophages (Fig. 4D). We further investigated whether pretreatment with FWG inhibits the activation of NF-κB signaling in DSS-induced colitis mice. In agreement with our in vitro results, pretreatment with FWG prevented DSS-induced NF-κB phosphorylation in colon tissues (Fig. 4E). These data showed that FWG suppressed the NF-κB signaling pathway by inhibiting the translocation of NF-κB.

DISCUSSION

Many studies have reported that various herbal medicines such as *aloe vera* gel, *Boswellia serrata* and *wheat grass juice* seem to be effective in the treatment of IBD (22). However, available evidence regarding herbal medicine is complex and incomplete, therefore, there is a need for further investigation to maximize the quality and efficacy. Ginseng is wildly used for traditional medicines. Ginsenosides, the major components of ginseng, have been reported to have a range of pharmacological and therapeutic effects such as anti-inflammation and anti-cancer activities. Recently, some studies presented evidence that ginseng has therapeutic effects on inflammation associated with IBD (23). However, the bioavailability of ginsenosides in humans after oral administration of ginseng is very low (24). Therefore, many studies have suggested methods such as mild acid hydrolysis as well as enzymatic and microbial conversion to improve the bioactivity and bioavailability of ginsenosides (25). Consequently, we developed fermented wild ginseng through enzymatic conversion (fermentation), which is a highly selective method that utilizes *Aspergillus niger*, *Aspergillus oryzae* and *Penicillum sp* derived enzymes. Based on our HPLC analysis, we found that our fermentation process could convert the major ginsenosides, Re, Rg1, Rg2, Rc, and Rb2 into ginsenoside metabolites, Rh1, F1, C-K, PPT, and PPD which are active forms of the ginsenosides (Fig. 1A). It was recently reported that the conversion of Rb1, Rb2, and Rc into their deglycosylated metabolite C-K could considerable increase its bioactivity and bioavailability (26). In addition, Rg1 and Re are metabolized to Rh1 or F1, which are more easily absorbed into the bloodstream and act in target tissues (21). Among ginsenoside metabolites, C-K and Rh1 in particular are more active than other metabolites against inflammation (25, 27). There is already a report that C-K suppresses the inflammatory response and has therapeutic effects in DSS-induced colitis (28). This suggests that FWG may be biochemically and functionally superior to raw material, especially against inflammation.

To investigate the anti-inflammatory effects of FWG in IBD, we used a DSS-induced colitis mouse model, which has symptoms similar to human colitis. Our data showed that FWG treatment significantly suppressed DSS-induced colonic inflammation and crypt damage (Fig. 2B). Some studies suggest that activation of intestinal macrophages may be involved in the major pathway of IBD (29). Activated macrophages are

considered major mediators in the production of pro-inflammatory cytokines such as IL-1β, IL-6, IL-12 p40, and TNF-α in the gut. Excessive production of cytokines is the pathogenesis for IBD (30). For this reason, the regulation of inflammatory cytokines production has been largely studied and is even regarded as a new therapeutic target for the treatment of IBD. We observed an increase in the levels of pro-inflammatory cytokines in the colon tissue of DSS-induced mice and FWG treatment reduced the expression of pro-inflammatory cytokines at the mRNA and protein levels (Fig. 2A, B). Furthermore, FWG suppressed DSS-induced macrophage infiltration (Fig. 2C).

IBD is related to damages in the intestinal barrier, which regulates the infiltration of endotoxins or gram-negative bacteria (31). Tight junction complexes support the intestinal epithelial barrier and are formed by transmembrane and intracellular proteins such as ZO-1 (32, 33). Therefore, we investigated the distribution and expression of the tight junction protein, ZO-1. As previously reported (19), we observed the loss of ZO-1 expression in DSS-induced mice. Interestingly, treatment with FWG recovered the expression and distribution of ZO-1 in the colon of DSS-induced mice (Fig. 2D), suggesting that the regulation of colitis by FWG might be due to improvements in the function of the intestinal epithelial barrier.

A number of studies have reported that NF-κB is substantially activated in DSS-induced colitis and in patients with IBD, and the levels of activated NF-κB are associated with the severity of inflammation (21). The activation of NF-κB causes the production of inflammatory mediators including the recruitment of pro-inflammatory cytokines and the activation of immune cells such as macrophages. This suggests that blocking NF-κB signaling might be a potential target for the treatment of IBD (32). We found that FWG exerts inhibitory effects on these inflammatory responses via the suppression of NF-κB activation in DSS-induced mice and LPS-stimulated RAW264.7 macrophages (Fig. 4), suggesting that the anti-colitic effects of FWG may be related to the inhibition of macrophage activation via NF-κB.

In conclusion, the present study demonstrated the anti-inflammatory activity and regulation of intestinal barrier by FWG in DSS-induced mice. Accordingly, we conclude that FWG can be a useful compound for the prevention and treatment of IBD.

MATERIALS AND METHODS

Reagents and Materials
DSS (molecular weight 36-50 kDa) was purchased from MP Biomedical (OH, USA). DMEM, RPMI 1640, fetal bovine serum (FBS), penicillin (100 unit/ml) and streptomycin (100 μg/ml) were obtained from Welgene Inc. (Daegu, Korea). LPS purified from Escherichia coli O111:B4 was purchased from Sigma-Aldrich (MO, USA). The following antibodies were used in these studies: p-p65 (Cell signaling, MA, USA), IκB α (Cell signaling, MA, USA), p65 (Santa Cruz, TX, USA), ZO-1 (Invitrogen,

CA, USA) and TNF α- PE (BD Biosciences, CA, USA).

Preparation of fermented wild ginseng (FWG)
Cultured wild ginseng root, generated from tissue cultures of Korean wild ginseng, was purchased from Vitrosis Co., LTD. (Young-ju, Korea) in Nov 2013. The wild ginseng was completely pulverized (particle size below 1,000 μm). The ginseng powder was incubated with cellulase and hemi-cellulase at 60°C for 6 h and then pretreated in a pressurized condition at 125°C for 30 min. The pretreated powder was fermented through the process of enzyme reaction with lactase, beta-glucosidase, and naringinase derived from *Aspergillus niger*, *Aspergillus oryzae* and *Penicillum sp*. The product was pulverized again to particle sizes below 1 μm. The fermented wild ginseng powder (FWG) was sterilized using an autoclave at 121°C for 15 min. For treatment, the FWG was dissolved in DMSO and filtered with a syringe filter (pore size 0.22 μm).

HPLC analysis
To measure the changes in ginsenoside composition in fermented wild ginseng, HPLC analysis was carried out on an Agilent 1200 series (Agilent, Santa Clara, CA, USA) LC system equipped with a separation module with an integrated column heater, an autosampler, and a photodiode array detector. UV absorbance was monitored at 200 to 400 nm. Quantification was performed by integrating the peak areas at 203 nm. The injection volume was 10 μl. A column (Thermo Hypersil gold ODS-H80, 150 × 4.6 mm; particle size, 5 um; Thermo Electron Corporation, Cheshire, UK) was installed in a column oven and maintained at 45°C. The mobile phase was composed of water (solvent A) and acetonitrile (solvent B). The flow rate was 1.0 ml/J. The gradient was 0.0 min, 5% B; 10 min, 5% B; 40.0 min, 32% B; 55.0 min, 60% B; 70.0 min, 75.0% B; 72.0 min, 90.0% B; 82.0 min, 90.0% B; 84.0 min, 5.0% B and 90.0 min, 5% B.

Cell culture
RAW264.7 murine macrophages were obtained from the Korea Cell Line Bank (Seoul, Korea). Cells were grown at 37°C in RPMI supplemented with 10% FBS, penicillin (100 units/ml) and streptomycin (100 μg/ml) in a humidified atmosphere of 5% CO_2.

Experimental animal
Six-week old female C57BL/6J mice were received from the Orient Co. (Charles River Laboratories). All animal procedures were performed according to the approved protocols [LCDI-2013-0045] and institution recommendations for the proper use and care of laboratory animals, Center of Animal Care and Use, College of Pharmacy, Gachon University.

Induction of colitis
Experimental colitis was induced by exposing mice to drinking water containing 2% (w/v) DSS for 7 days. For each experi-

ment, the mice were divided into 3 groups. The first group was the vehicle-treated control, and the second group was given drinking water with DSS only. The last group consisted of mice pretreated with 100 mg/kg FWG (5 times a week, P.O.) for 3 weeks, and then 2% DSS with 100 mg/kg FWG for 1 week according to the experimental design. The experiments were conducted twice, with 6 mice in each group.

Histological analysis of colon

Paraffin-embedded tissue sections of swiss-rolled whole colon were stained with hematoxylin and eosin for histological scoring. Samples from the entire colon were examined in a blinded fashion. Colitis assessment was based on criteria including the degree of inflammation (scale of 0-3), edema (0-4) and crypt damage (0-4), as described previously (34).

RNA isolation and RT-PCR

Total RNA was isolated using TRIzol reagent (Invitrogen, CA, USA), according to the manufacturer's protocol. First strand cDNA was synthesized with the PrimeScript RT reagent Kit (TaKaRa, Shiga, Japan) using 3 μg total RNA according to the manufacturer's protocol. cDNA was then amplified by PCR with mouse specific primers: IL-1β, GCC TTG GGC CTC AAA GGA AAG AAT C (forward) and GGA AGA CAC AGA TTC CAT GGT GAA G (reverse); IL-6, TGG AGT CAC AGA AGG AGT GGC TAA G (forward) and TCT GAC CAC AGT GAG GAA TGT CCA C (reverse); IL-12p40, GTC CTC AGA AGC TAA CCA TC (forward) and TTT CCA GAG CCT ATG ACT CC (reverse); TNF-α, ATA GCT CCC AGA AAA GCA AGC (forward) and CAC CCC GAA GTT CAG TAG ACA (reverse); INFγ: AGC GGC TGA CTG AAC TCA GAT TGT AG (forward) and GTC ACA GTT TTC AGC TGT ATA GGG (reverse); β-actin: TGG AAT CCT GTG GCA TCC ATG AAA C (forward) and TAA AAC GCA GCT CAG TAA CAG TCC G (reverse). The RT-PCR exponential phase was determined on cycles 28-33 to allow quantitative comparisons among the cDNAs amplified from identical reactions.

Western blot analysis

Whole cell, cytoplasmic and nuclear extracts were prepared as described previously (35). Protein extract lysates were resolved by SDS-polyacrylamide gel electrophoresis and transferred to polyvinylidene fluoride membranes. The protein-antibody complexes were detected by Absignal (Abclone, Seoul, Korea) according to the manufacturer's recommended protocol.

Analysis of flow cytometry for intracellular cytokine detection

For intracellular cytokine staining, RAW264.7 were resuspended in PBS containing 0.05% Tween 20 and incubated for 30 min. After washing in PBS, cells were incubated with anti-mouse TNF α-PE with 0.02% Tween 20 solution for 30 min on ice. Cells were subjected to flow cytometry analysis on a FACSCalibur cytometer (BD Biosciences, CA, USA). 10,000 events were acquired and analyzed using the Flowzo data analysis software (Tree Star, OR, USA).

Immunofluoresence assay

In RAW264.7, cells grown on glass coverslips were fixed and made permeable in methanol at −20°C. The coverslips were incubated with anti-p65 antibody overnight at 4°C. Colon sections were fixed with 4% para-formaldehyde for 1 h then stained with primary antibody overnight at 4°C followed by incubation in FITC-labeled secondary antibody for 1 h at room temperature. Sections and coverslips were then mounted with mounting medium containing 4,6-diamidino-2-phenylindole (DAPI) for nuclear counterstaining. Images were observed by fluorescence microscopy. FITC and DAPI images were obtained from the same area.

Statistical analysis

The results are expressed as mean ± SD values of triplicate experiments. Each treated group was compared with the control group and statistical significance was determined by Student's t tests. Values with $P < 0.001$ were considered significant.

ACKNOWLEDGEMENTS

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