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Ginsenoside Rb1 and its metabolite compound K inhibit IRAK-1 activation—The key step of inflammation

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ABSTRACT

In the preliminary study, ginsenoside Rb1, a main constituent of the root of *Panax ginseng* (family Araliaceae), and its metabolite compound K inhibited a key factor of inflammation, nuclear transcription factor κB (NF-κB) activation, in lipopolysaccharide (LPS)-stimulated murine peritoneal macrophages. When ginsenoside Rb1 or compound K were orally administered to 2,4,6-trinitrobenzene sulfuric acid (TNBS)-induced colitic mice, these agents inhibited colon shortening, macroscopic score, and colonic thickening. Furthermore, treatment with ginsenoside Rb1 or compound K at 20 mg/kg inhibited colonic myeloperoxidase activity by 84% and 88%, respectively, as compared with TNBS alone ($p < 0.05$), and also potently inhibited the expression of tumor necrosis factor-α, interleukin (IL)-1β and IL-6, but increased the expression of IL-10. Both ginsenoside Rb1 and compound K blocked the TNBS-induced expressions of COX-2 and iNOS and the activation of NF-κB in mice. When ginsenoside Rb1 or compound K was treated in LPS-induced murine peritoneal macrophages, these agents potently inhibited the expression of the proinflammatory cytokines. Ginsenoside Rb1 and compound K also significantly inhibited the activation of interleukin-1 receptor-associated kinase-1 (IRAK-1), IKK-β, NF-κB, and MAP kinases (ERK, JNK, and p-38); however, interaction between LPS and Toll-like receptor-4, IRAK-4 activation and IRAK-2 activation were unaffected. Furthermore, compound K inhibited the production of proinflammatory cytokines more potently than did those of ginsenoside Rb1. On the basis of these findings, ginsenosides, particularly compounds K, could be used to treat inflammatory diseases, such as colitis, by targeting IRAK-1 activation.

1. Introduction

Many gram-negative bacteria residing in the intestines of humans and animals synthesize endotoxins, such as, lipopolysaccharides (LPS) that resemble those found in *Escherichia coli* [1]. Most types of LPS are detected at picomolar levels by an ancient receptor of the innate immune system present on the macrophages and endothelial cells of animals [2]. LPS activate the biosynthesis of diverse mediators of inflammation, such as tumor necrosis factor (TNF)-α, interleukin (IL)-1β, and IL-6, via a toll-like receptor (TLR)-4-linked nuclear transcription factor κB (NF-κB) pathway in macrophages and activates the production of co-stimulatory molecules required for the adaptive immune response [3]. TLR-4, which is linked to the activation of transcription factor NF-κB via interleukin-1 receptor-associated kinases (IRAKs), serves as the main mediator of intestinal bacterial LPS signaling in intestinal bowel disease [4,5]. IRAKs are protein kinases involved in the signaling of innate immune responses from TLRs. After TLR-4 recognizes pathogen-associated molecular patterns, such as LPS, all IRAK members form multimeric receptor complexes [6]. In particular, phosphorylated IRAK-1 is then degraded via an ubiquitin-dependent mechanism and activates a multimeric protein complex composed of TRAF6, TAK1, TAB1, and TAB2. Activated TAK1 phosphorylates both the IKKs and specific MKKs. IKKs phosphorylate the NF-κB inhibitor IκB-α, leading to its ubiquitination and subsequent degradation by the proteasome. This degradation of IκB-α allows NF-κB to translocate to the nucleus and bind to specific promoter sequences. On the other hands, activated MKKs phosphorylate and activate members of the JNK/p38 MAP kinase (MAPK) family [7]. Furthermore, the activation of NF-κB in mucosal macrophages is accompanied by the increased production and secretion of IL-1β, TNF-α, and IL-6, by the cells, but a decrease in IL-10 expression.

Thus, these mediators stimulate the innate immune response but their overexpression may cause endotoxemia, leading to tissue injury, organ failure, shock, and even death [8]. Therefore, regulating the expression of these inflammatory mediators could be therapeutically useful for treating inflammatory diseases.

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Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae), which contains ginsenoside Rb1 as its main constituent, is frequently used as a traditional medicine in Asian countries [9]. The ginsenosides, including ginsenoside Rb1, have been reported to have various biological activities, including anti-inflammatory action and anti-tumor effects (inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis) [10,11]. Orally administered ginsenoside Rb1 is metabolized to 20-*O*-β-D-glucopyranosyl-20(S)-protopanaxadiol (named as compound K) by intestinal bacteria before its absorption to the blood, and the metabolite, compound K, is absorbed from the gastrointestinal tract to the blood [12–14]. Compound K has many pharmacological activities, including anti-tumor, anti-diabetic, anti-inflammatory, and anti-allergic effects [15–17]. However, the anti-inflammatory mechanism of ginsenoside Rb1 and its metabolite, compound K, has not been thoroughly studied.

Therefore, in the present study, we studied the anti-inflammatory effects of ginsenoside Rb1 and compound K and the mechanisms responsible in mice with TNBS-induced colitis and in LPS-stimulated peritoneal macrophages.

2. Materials and methods

2.1. Materials

Ginsenoside Rb1 and compound K (Fig. 1) were isolated using the previously published method of Bae et al. [18]. RPMI 1640, sulfasalazine, 2,4,6-trinitrobenzene sulfonic acid (TNBS) and LPS purified from *E. coli* O111:B4 and penicillin-streptomycin were purchased from Sigma–Aldrich (St. Louis, MO, U.S.A.). Antibodies for ZO-1, Claudin-1, TLR-4, IRAK-1, IRAK-2, IRAK-4, COX-2, iNOS, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, LA, U.S.A.). Antibodies for p-IRAK-1, p-IKK-β, p-IκB-α, p-p65, p65, p-p38, p38, p-JNK, JNK, p-ERK, and ERK were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). Cytokine enzyme-linked immunosorbent assay (ELISA) kits were purchased from R&D Systems (Minneapolis, MN, U.S.A.). Other chemicals were of the highest commercial grade available.

2.2. Animals

Male ICR mice (20–25 g, 5 weeks old) were supplied by the Oriental Animal Breeding Center (Sungnam, Korea). All animals were housed in wire cages at 50 ± 10% humidity and 20–22 °C, fed standard laboratory chow (Samyang Co., Seoul, South Korea), and allowed water ad libitum. All experiments were performed in accordance with the NIH and Kyung Hee University guidelines for Laboratory Animals Care and Use, and the study was approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University.

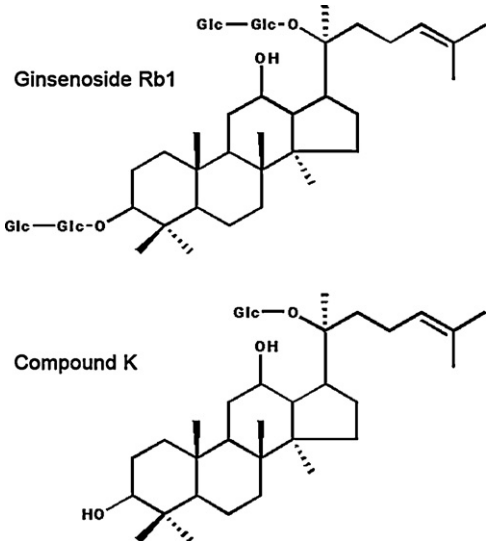

Fig. 1. The structures of ginsenoside Rb1 and compound K.

2.3. Preparation of the experimental colitic mouse model

The curative effects of ginsenoside Rb1 and compound K were investigated in male ICR mice. Mice were divided into seven groups, that is, normal control and TNBS-induced colitic groups treated with or without ginsenoside Rb1 (10 or 20 mg/kg), compound K (10 mg/kg or 20 mg/kg) or sulfasalazine (50 mg/kg). TNBS-induced colitis was induced by the intrarectal 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 [19]. The normal control 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 agents within the entire colon and cecum, mice were held in a vertical position for 30 s after the injection. Using this procedure, >96% of the mice retained the TNBS enema. If an animal quickly excreted the TNBS–ethanol solution, it was excluded from the remainder of the study. Ginsenoside Rb1, compound K (10 or 20 mg/kg) or sulfasalazine (50 mg/kg) was orally administered once a day from one day after TNBS treatment to the day before sacrifice for 3 days. Mice were sacrificed 12 h after the final administration of test agents and colons were quickly removed, opened longitudinally, and gently cleared of stool with PBS. Macroscopic assessment of the disease grade was scored according to a previously reported scoring system (0, no ulcer and no inflammation; 1, ulceration and local hyperemia; 2, ulceration without 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) [20], and the colon tissue was then used for immunoblot and ELISA analysis, as described below.

For the histological examinations, the colons were fixed in 10%-buffered formalin solution, embedded in paraffin using standard methods, cut into 5-μm sections, stained with hematoxylin–eosin, and then assessed under an optical microscope.

2.4. Immunostaining for myeloperoxidase

Neutrophils were immunolocalized using a 3-step staining procedure consisting of sequential incubation with 1st and 2nd antibodies and detected using DAB substrate kit from Thermo scientific (Rockford, IL, U.S.A.). Inflammatory cell profiles in the colonic tissues were investigated using anti-neutrophil [myeloperoxidase (MPO), CD66b, and neutrophil elastase] antibodies. The serial sections were subjected to this procedure. Horseradish peroxidase activity was visualized with 3-amino-9-ethylcarbazole.

2.5. Assay of myeloperoxidase activities in colon tissues

Colons were homogenized in a solution containing 0.5% hexadecyl trimethyl ammonium bromide dissolved in 10 mM potassium phosphate buffer (pH 7.0), and then centrifuged at 20,000 × *g* for 20 min at 4 °C. An aliquot (50 μL) of the supernatant was then 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 monitored at 650 nm over time. Myeloperoxidase activity was defined as the quantity of enzyme degrading

1 μmol/mL of peroxide at 37 °C and expressed in unit per milligram protein. Protein contents were assayed using the Bradford method [21].

2.6. ELISA and immunoblotting of TNBS-induced mice

For the ELISA of IL-1β, TNF-α, IL-6 and IL-10, colons were homogenized in 1 mL of ice-cold RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. Lysates were centrifuged (15,000 × *g*, 4 °C) for 10 min, and supernatants were transferred to 96-well ELISA plates. IL-1β, TNF-α, IL-6 and IL-10 concentrations were determined using commercial ELISA kits. For immunoblot analysis of ZO-1, Claudin-1, iNOS, phospho-NF-κB (pp65), NF-κB (p65), cyclooxygenase (COX)-2 and β-actin, colon tissues were carefully homogenized to obtain viable single cells, which were then resuspended in 1 mL of RIPA lysis buffer containing 1% protease inhibitor cocktail and 1% phosphatase inhibitor cocktail. After centrifugation, supernatants were immunoblotted. Proteins from collected cells were electrophoresed in 9% sodium dodecyl sulfate polyacrylamide gel and then transferred to nitrocellulose membrane. Levels of pp65, p65, COX-2, iNOS and β-actin were assayed as previously described [22]. Immunodetection was performed using an enhanced chemiluminescence detection kit.

2.7. Isolation and culture of peritoneal macrophages

Male ICR mice were intraperitoneally injected with 2 ml of 4% thioglycolate solution, and sacrificed four days after injection. Peritoneal cavities were flushed with 10 ml of RPMI 1640 and lavage fluids were centrifuged at 300 × *g* for 10 min. The prepared cells were resuspended in RPMI 1640, plated, incubated for 1.5 h at 37 °C, washed three times and nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates (0.6×10^6 cells/well) at 37 °C in RPMI 1640 containing 10% FBS. Attached cells were used as peritoneal macrophages [23].

2.8. Determination of cytokine levels in LPS-induced peritoneal macrophage

Cytokines, TNF-α and IL-1β, were assessed using commercially available ELISA kits according to the manufacturer's instructions.

2.9. Immunoblotting of peritoneal macrophage

The cell supernatant extracts prepared from macrophages were separated by 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes. The membranes were blocked with 5% non-fat dried-milk proteins in PBST, then probed with TLR-4, iNOS, COX-2, IRAK-1, IRAK-2, IRAK-4, p-IKK-β, p-p65, p-p38, p38, p-JNK, JNK, p-ERK, ERK, ubiquitin or β-actin antibody. They were then washed with PBST, and proteins were detected using HRP-conjugated secondary antibodies for 1 h. Bands were visualized with enhanced chemiluminescence (ECL) reagent [22].

2.10. Immunoprecipitation

Mouse peritoneal macrophages were cultured in 6-well plates in RPMI 1640 with or without ginsenoside Rb1 or compound K for 1 h and then treated with or without 50 ng/ml LPS for 30 min. The cells were lysated with 300 μL of lysis buffer per 100-mm culture dish. Lysates (3 ml) were supplemented with 9 ml of NET buffer [50 mM Tris–HCl (pH 7.4), 150 mM NaCl, 5 mM EDTA, and 0.05% Nonidet P-40] and incubated overnight at 4 °C with 10 μL of IRAK-1 antibody (200 μg/ml). To precipitate immune complexes, lysates were incubated with 50 μL of protein A/G PLUS-Agarose (Santa Cruz, LA, U.S.A.) for 1 h at 4 °C. Bead-bound complexes were washed three times with cold NET buffer and denatured for 10 min at 100 °C, centrifugated and immunoblotted.

2.11. Immunofluorescent confocal microscopy

To confirm the nuclear translocation of NF-κB, peritoneal macrophages were stimulated with LPS (50 ng/ml) in the presence or absence of ginsenoside Rb1 or compound K (10 μM) for 60 min. The cells were then fixed with 4% formaldehyde and permeabilized with 0.2% Triton X-100. Cells were stained with goat polyclonal anti-p65 antibody for 2 h at 4 °C, incubated with secondary antibodies conjugated with Alexa 488 and propidium iodide (10 μg/ml, Calbiochem Co., San Diego, CA, U.S.A.) for 1 h, and observed under a confocal microscope.

To assay LPS/TLR-4 complex formation, peritoneal macrophages were plated on cover slides, incubated at 37 °C overnight, and stimulated with Alexa Fluor 594-conjugated LPS (10 μg/ml, Invitrogen, CA, U.S.A.) for 20 min in the presence or absence of ginsenoside Rb1 or compound K. They were then fixed with 4% formaldehyde and 3% sucrose for 20 min [24], stained with rabbit polyclonal anti-pTLR-4 antibody for 90 min at 4 °C, and incubated with secondary antibodies conjugated with Alexa Fluor 488 for 1 h. Stained cells were observed under a confocal microscope.

To assay of IRAK-1, peritoneal macrophages were plated on cover slides were incubated at 37 °C overnight, stimulated with LPS (100 ng/ml) for 30 min in the presence or absence of ginsenoside Rb1 or compound K, fixed with 4% formaldehyde, permeabilized with 0.2% Triton X-100, stained with goat polyclonal anti-IRAK-1 antibody for 2 h at 4 °C and then incubated with secondary antibodies conjugated with Alexa 488 for 1 h. Stained cells were observed under a confocal microscope.

2.12. Transient transfection of small interfering RNA (siRNA)

Cells were seeded at 3×10^5cells/well in 24-well plates and allowed to rest for one day prior to the transfection. They were then transfected with 50 nM siRNA for IRAK-1 using Lipofectamine™ 2000 (Invitrogen, Carlsbad, CA) according to the manufacturer's instruction. At 24 h after transfections, cells were treated with or without ginsenoside Rb1 or compound K with or without LPS for 30 min.

2.13. IRAK-1 kinase activity assay

IRAK-1 kinase activity was assayed by using a LanthaScreen™ Eu Kinase Binding Assay kit (Invitrogen, Carlsbad, CA, U.S.A.). To evaluate the inhibitory effects of ginsenoside Rb1 and compound K, test agents (ginsenoside Rb1, compound K, or staurosporine) in 1 × Kinase Buffer A (Invitrogen, Carlsbad, CA, U.S.A.) was incubated in a reaction mixture (total volume in 96 wells, 60 μL) containing 5 nM IRAK-1, 2 nM Eu-Anti-GST antibody, 100 nM KinaseTracer 236[2] for 1 h at 25 °C. Fluorescence (340 and 665 nm; 340 and 615 nm) was then measured using a fluorescence multiplate reader (Bio-Rad Laboratories, Hercules, CA, U.S.A.).

2.14. Statistical analysis

Results are presented as the means ± standard deviation of at least three replicates. ANOVA was used for comparisons between multiple groups. The Student *t*-test was used for the statistical analysis of the difference noted. *P* values of 0.05 or less were considered statistically significant.

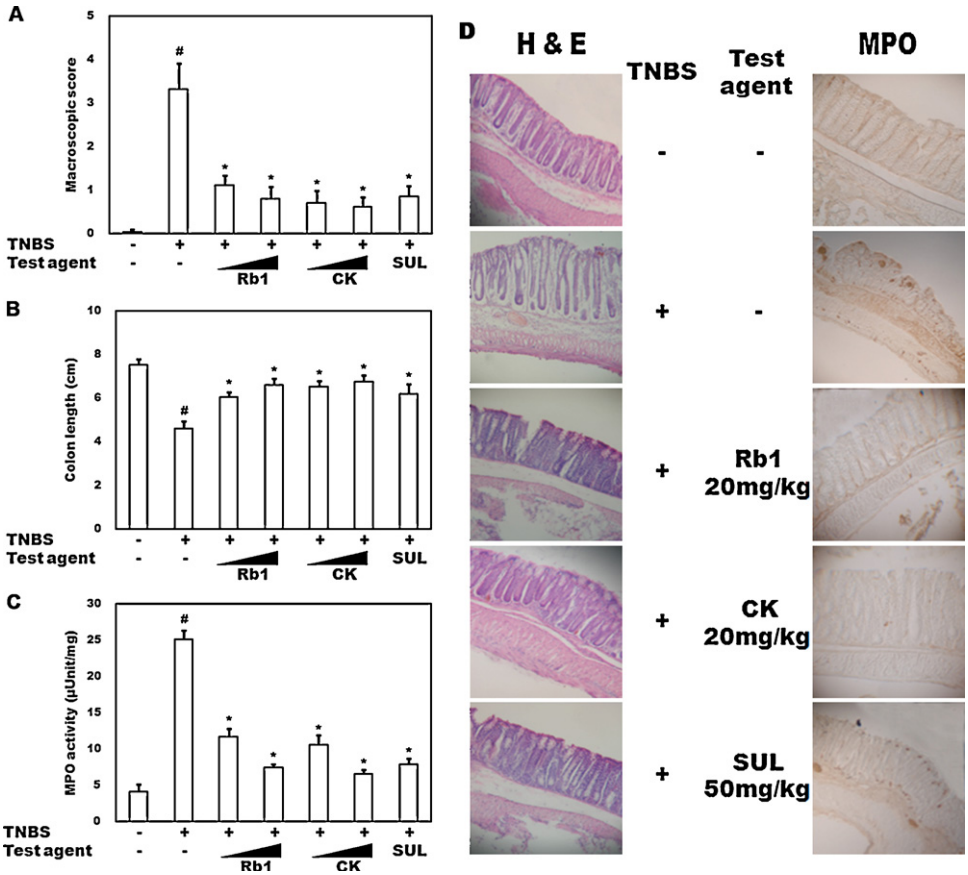

Fig. 2. Inhibitory effects of ginsenosides in TNBS-induced colitic mice. The effects of ginsenoside Rb1 and compound K on macroscopic disease (A), colon length (B), colonic MPO activity (C), histology (hematoxylin–eosin staining), and myeloperoxidase (MPO) immunostaining (D) in TNBS-induced colitic mice. TNBS, except in the normal control group, was intrarectally administered to mice treated with saline, ginsenoside Rb1, compound K or sulfasalazine. Ginsenoside Rb1 (Rb1) and compound K (CK) (10 mg/kg or 20 mg/kg), sulfasalazine (SUL, 50 mg/kg), or saline was orally administered for 3 days after TNBS treatment. Mice were sacrificed 12 h after the final administration of test agents. All values are means ± SDs ($n = 7$). $^{\#}p < 0.05$, significantly different vs. normal control group; $^{*}p < 0.05$, significantly different vs. TNBS group.

3. Results

3.1. Ginsenoside Rb1 and compound K inhibits pro-inflammatory cytokines and NF-κB activation in TNBS-induced colitic mice

We tested the curative effects of ginsenoside Rb1 and compound K to inhibit the colitis induced by TNBS in mice. TNBS caused a loss of body weight and severe inflammation manifested by shortened, thickened, and erythematous colons. Histologic examinations of excised colon tissues revealed increased neutrophil numbers, massive bowel edema, dense infiltration of the superficial mucosal layers, and epithelial cell disruption due to large ulcerations. To investigate the curative effect of ginsenoside Rb1 or compound K, these agents were orally administered for 3 days, from 1 day after TNBS treatment. Treatment with ginsenoside Rb1 or compound K inhibited colon shortening, inflammation, and thickening (Fig. 2). Treatment with ginsenoside Rb1 and compound K at a dose of 20 mg/kg inhibited colonic MPO activity by 84% and 88%, respectively, compared with TNBS alone ($p < 0.05$; Fig. 2C). Expression of tight junction-associated proteins, that is, ZO-1 and Claudin-1, decreased in TNBS-treated mice. However, ginsenoside Rb1 and compound K inhibited the decrease in tight junction-associated proteins (Fig. 3). Ginsenoside Rb1 and compound K were more potent inhibitors than was sulfasalazine, which is used to treat colitis.

We next measured levels of the pro-inflammatory cytokines IL-1β, TNF-α, and IL-6 and proinflammatory cytokine IL-10 in the colon tissues of TNBS-induced colitic mice (Fig. 4). TNBS increased the expression of IL-1β, TNF-α and IL-6 by 6.3-fold, 21.3-fold, and 3.4-fold, respectively, but inhibited IL-10 expression by 75%. However, treatment with ginsenoside Rb1 or compound K inhibited the TNBS-induced expression of these cytokines, but did not affect β-actin expression. Treatment with ginsenoside Rb1 and compound K at 20 mg/kg inhibited the expression of these cytokines by 73% and 82% ($p < 0.05$), 70% and 84% ($p < 0.05$), and 80% and 85% ($p < 0.05$), respectively, but increased IL-10 expression by 45% and 53%, respectively ($p < 0.05$). TNBS also increased

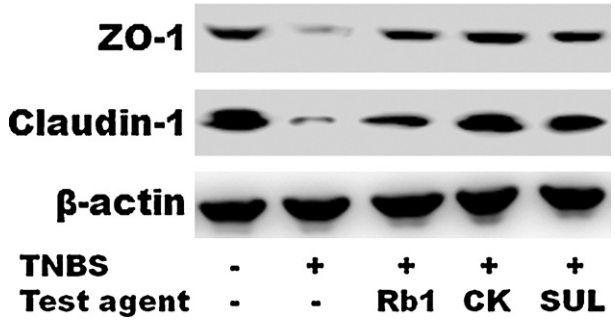

Fig. 3. Effects of ginsenoside Rb1 and compound K on the expressions of tight junction-associated proteins in TNBS-induced colitic mice. TNBS, except in the normal control group, was intrarectally administered to mice treated with saline, ginsenoside Rb1 (Rb1, 20 mg/kg), compound K (CK, 20 mg/kg), or sulfasalazine (SUL, 50 mg/kg). Test agents were orally administered for 3 days after TNBS treatment. Mice were anesthetized and sacrificed 12 h after the final administration of test agents. The colons of the mice were collected, and ZO-1 and claudin-1 expressions were measured by immunoblot analysis.

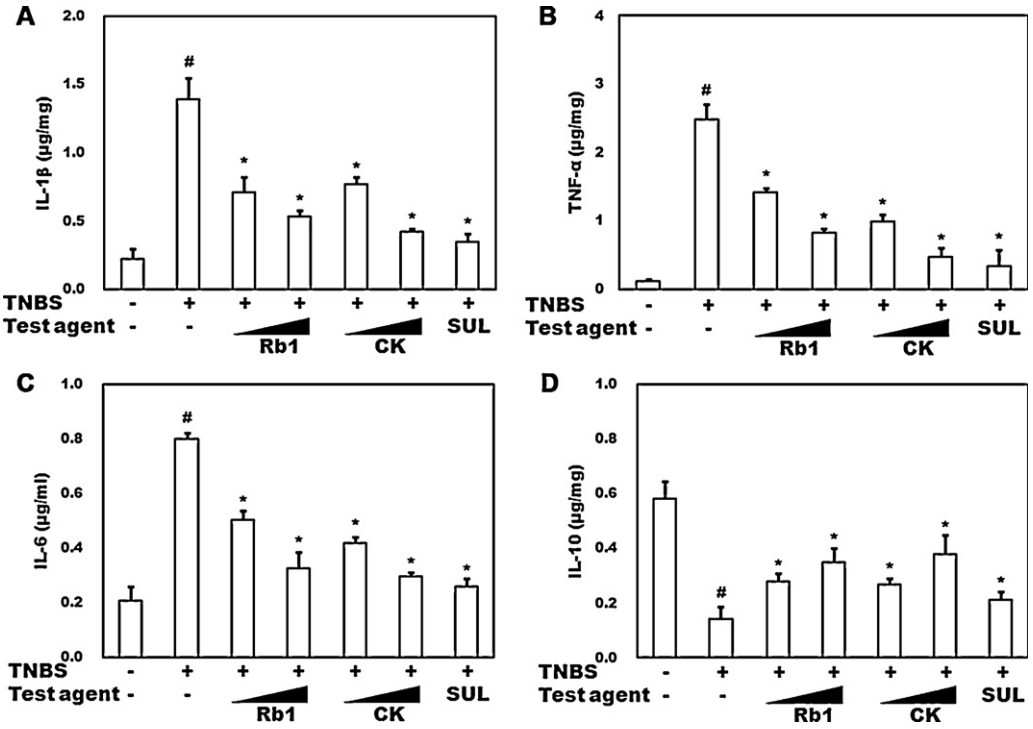

Fig. 4. Effects of ginsenoside Rb1 and compound K on inflammatory cytokines in TNBS-induced colitic mice. TNBS, except in the normal control group, was intrarectally administered to mice treated with saline, ginsenoside Rb1, compound K or sulfasalazine. Ginsenoside Rb1 (Rb1) and compound K (CK) (10 mg/kg or 20 mg/kg), sulfasalazine (SUL, 50 mg/kg) or saline was orally administered for 3 days after TNBS treatment. Mice were anesthetized and sacrificed 12 h after the final administration of test agents. Colons were collected and IL-1β (A), TNF-α (B), IL-6 (C) and IL-10 (D) expressions were measured by ELISA. All values are means ± SDs ($n = 7$). $^{\#}p < 0.05$, significantly different vs. normal control group; $^{*}p < 0.05$, significantly different vs. TNBS group.

the expressions of COX-2, iNOS, and NF-κB (pp65). Ginsenoside Rb1 and compound K blocked the induction of COX-2, iNOS, and pp65 by TNBS. Ginsenoside Rb1 and compound K were more potent than sulfasalazine (Fig. 5).

3.2. Effects of ginsenoside Rb1 and compound K on the protein expressions of pro-inflammatory cytokines in LPS-induced peritoneal macrophages

To investigate the anti-inflammatory effects of ginsenoside Rb1 and compound K in peritoneal macrophage cells, we measured the inhibitory effect on the protein expressions of pro-inflammatory cytokines, IL-1β and TNF-α. Stimulation of peritoneal macrophages with LPS increased the expressions of IL-1β and TNF-α. When cells were treated with LPS in the presence of ginsenoside Rb1 or compound K, the expression of IL-1 and TNF-α was significantly decreased (Fig. 6). No cytotoxic effects of ginsenoside Rb1 and compound K were observed in the cell viability test (crystal violet method) under the conditions used in these experiments (data not shown).

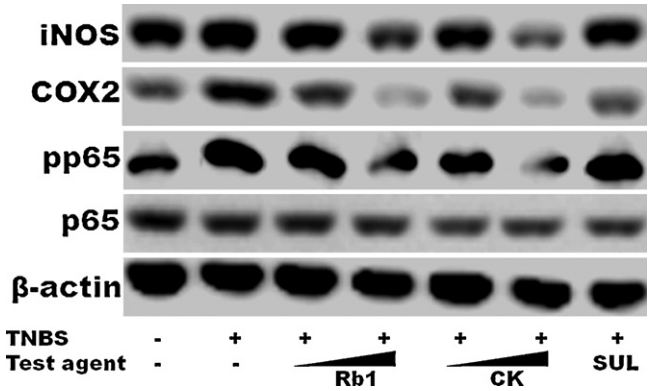

Fig. 5. Effect of ginsenoside Rb1 and compound K on iNOS and COX-2 expression and NF-κB activation in TNBS-induced colitic mice. TNBS, except in the normal control group, was intrarectally administered to mice treated with saline, ginsenoside Rb1, compound K or sulfasalazine. Ginsenoside Rb1 (Rb1) and compound K (CK) (10 mg/kg or 20 mg/kg), sulfasalazine (SUL, 50 mg/kg) or saline was orally administered from one day after TNBS treatment for 3 days. Mice were anesthetized and sacrificed 12 h after the final administration of test agents. Colons were collected and the expressions of iNOS and COX-2 expression and the activation of NF-κB were measured by immunoblot analysis.

3.3. Inhibitory effects of ginsenoside Rb1 and compound K on inflammatory enzyme production in LPS-stimulated peritoneal macrophages

Mouse peritoneal macrophages were stimulated with LPS in the presence or absence of ginsenoside Rb1 or compound K to determine whether ginsenoside Rb1 and compound K suppresses protein expression of COX-2 and iNOS, thus regulating the production of these proinflammatory mediators. We examined the protein levels of COX-2 and iNOS in peritoneal macrophages stimulated with LPS in the presence or absence of ginsenoside Rb1 or compound K (Fig. 7). LPS was found to induce both enzymes. However, ginsenoside Rb1 and compound K significantly inhibited the expression of these enzymes in LPS-stimulated cells.

3.4. Inhibitory effects of ginsenoside Rb1 and compound K on IKK-β phosphorylation, NF-κB activation, and IRAKs activation in LPS-stimulated peritoneal macrophages

The phosphorylation of IKK-β led to the nuclear translocation of NF-κB [25]. To investigate the inhibitory effects of ginsenoside Rb1 and compound K on the phosphorylation of IKK-β in LPS-induced peritoneal macrophages, cells were treated with LPS in the presence and absence of ginsenoside Rb1 or compound K

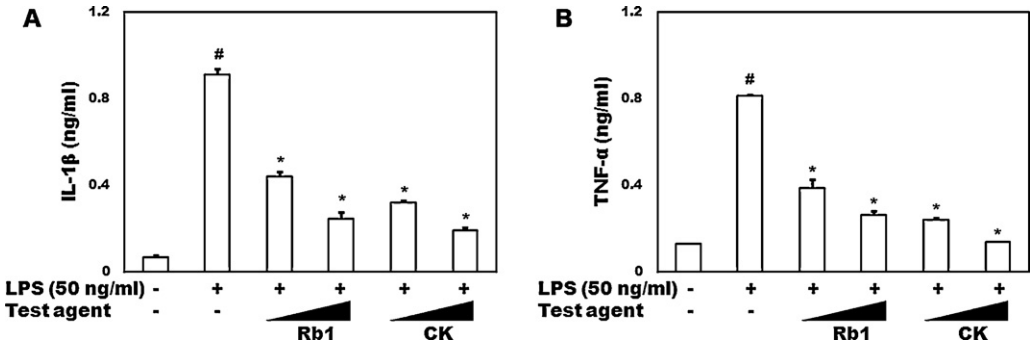

Fig. 6. Inhibitory effects of ginsenoside Rb1 and compound K on the productions of pro-inflammatory cytokines in LPS-induced peritoneal macrophages. Peritoneal macrophages (0.5×10^6 cells) were treated with 50 ng/ml LPS in the absence or presence of ginsenoside Rb1 (Rb1) or compound K (CK) (5 or 10 μM) for 20 h. Levels of IL-1β (A) and TNF-α (B) in culture supernatants were measured by ELISA. All data are expressed as means ± SDs ($n = 4$ in a single experiment). $^{\#}p < 0.05$, significantly different vs. normal control group.$^{*}p < 0.05$, vs. LPS control.

(Fig. 8A). Exposure to LPS increased the phosphorylations of IKK-β and IκB-α in cells. However, co-treatment with LPS and ginsenoside Rb1 or compound K reduced the LPS-induced phosphorylations of IKK-β and IκB-α.

Immunoblotting and confocal microscopy were used to determine whether ginsenoside Rb1 and compound K could inhibit the phosphorylation and the nuclear translocation of NF-κB in LPS-stimulated peritoneal macrophages (Fig. 8A and B). Cellular p-p65 levels and the nuclear translocation levels of p65 were increased in the macrophages treated with LPS alone. However, co-treatment with LPS and ginsenoside Rb1 or compound K inhibited the LPS-induced nuclear translocation level of p65 in the macrophages.

In addition, we investigated the effect of ginsenoside Rb1 and compound K on the LPS-induced the activations of MAPKs (ERK, JNK, and p38). LPS was found to activate MAPKs. However, ginsenoside Rb1 and compound K inhibited the activation of MAPKs (Fig. 8C). Of the MAPKs, pp38 was most potently inhibited.

To investigate the anti-inflammatory mechanism of ginsenoside Rb1 and compound K, we investigated the interaction between LPS and TLR-4 and measured IRAK-4 levels. When macrophages were treated with LPS in the presence or absence of ginsenoside Rb1 or compound K, LPS localized at the cell membrane. The LPS location was not affected by treatment with ginsenoside Rb1 or compound K (Fig. 8D). In addition, the expression level of IRAK-2 and IRAK-4 in LPS-stimulated macrophages was not influenced by ginsenosideRb1 or compound K (Fig. 8A).

Immunoblotting and confocal microscopy were then used to examine whether ginsenoside Rb1 and compound K inhibit the phosphorylation of IRAK-1, which leads to the phosphorylation of IKK-β [1]. Exposure to LPS was found to increase the phosphorylation-induced degradation and ubiquitination of IRAK-1 in peritoneal macrophages. However, treatment with ginsenoside Rb1 or compound K inhibited the phosphorylation-induced degradation and ubiquitination of IRAK-1 in LPS-treated cells (Fig. 8A, E and F).

To determine whether ginsenoside Rb1 and compound K inhibit IRAK-1 activation, we investigated their effects in IRAK-1 siRNA-treated murine peritoneal macrophages (Fig. 8G). Transfection with siRNA in macrophages significantly inhibited IRAK-1 expression by 80% compared with parental macrophages treated without siRNA. However, co-treatment with ginsenoside Rb1 or compound K plus LPS inhibited IRAK-1 degradation in siRNA-treated macrophages and parental macrophages. More specifically, co-treatment with compound K at 10 μM in the presence of LPS in siRNA-treated macrophages and parental macrophages inhibited IRAK-1 degradation by 79% and 85%, respectively, compared with in cells treated with LPS alone. However, neither ginsenoside Rb1 nor compound K reversed IKK-β phosphorylation in siRNA-treated or parental macrophages.

3.5. Inhibitory effects of ginsenoside Rb1 and compound K in IRAK-1 kinase binding activity

To determine whether ginsenoside Rb1 or compound K competitively inhibit IRAK-1, we used an IRAK-1 kinase binding activity assay kit. Ginsenoside Rb1 and compound K at 10 μM were found to inhibit IRAK-1 binding activity by 43% and 58%, respectively (Fig. 9). Their inhibitory effects are comparable with that of staurosporine (42% at 1 μM) (Fig. 9).

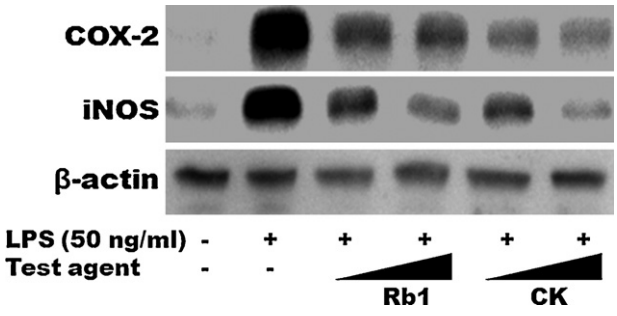

Fig. 7. Inhibitory effects of ginsenoside Rb1 and compound K on the expressions of COX-2 and iNOS in LPS-stimulated peritoneal macrophages. Peritoneal macrophages isolated from mice were incubated with LPS in the absence or presence of ginsenoside Rb1 (Rb1) or compound K (CK) (5 or 10 μM) for 20 h. Protein expressions of COX-2 and iNOS were measured by immunoblot analysis.

4. Discussion

Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae) is one the most widely used herbal medicines in East Asian countries. The major components of ginseng are ginsenosides, which are glycosides with a dammarane skeleton [26,27]. When ginseng and ginsenoside Rb1 are orally administered to humans or rats, compound K is detected in the blood and is believed to be produced by intestinal microflora in the intestine.

To confirm the metabolism of ginsenoside Rb1 by intestinal microflora, we orally administered ginsenoside Rb1 in normal and antibiotic-treated mice and analyzed the gastrointestinal tract for ginsenoside Rb1 and its metabolites (Supplement data 2). Ginsenoside Rb1 and its metabolite compound K were detected in the intestine of normal mice. However, compound K was not detected in the intestine of antibiotic-treated mice. Several *in vitro*

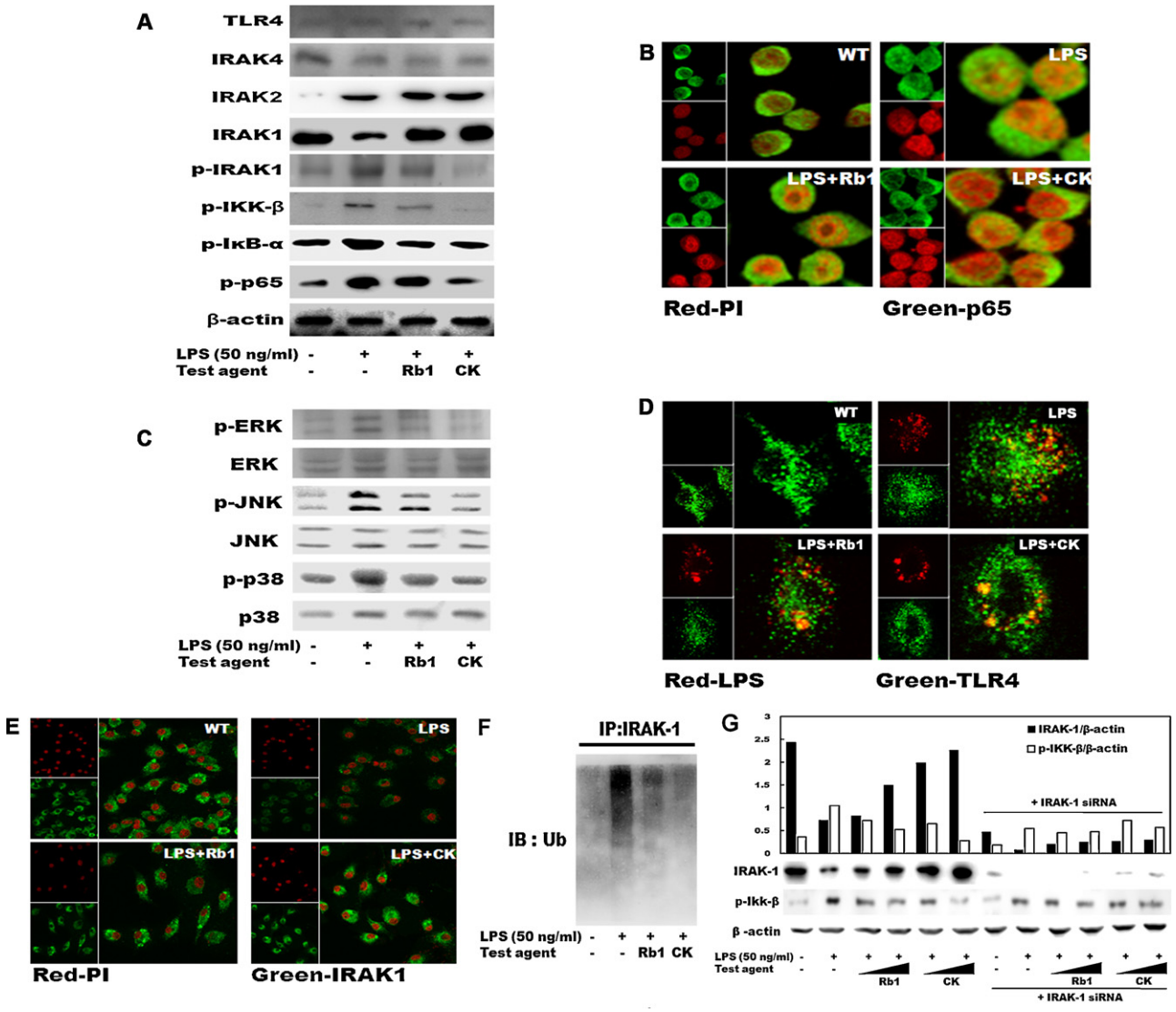

Fig. 8. Effects of ginsenoside Rb1 and compound K on phosphorylation of IKK-β, activations of NF-κB and IRAK-1, and interaction between LPS and TLR-4. Mouse peritoneal macrophages were treated with 50 ng/ml of LPS in the absence or presence of ginsenoside Rb1 (Rb1) or compound K (CK) (10 μM). (A) Effect on the phosphorylations of IKK-β, IκB-α, NF-κB and IRAK-1. They were determined 30 min after treatment with 50 ng/ml of LPS by immunoblot analysis. β-Actin was used as a control. (B) Effects on the nuclear translocation of NF-κB. They were detected by a confocal microscopy using an antibody for the p65 subunit. (C) Effects on the expressions of MAP kinases. Peritoneal macrophages were incubated with 100 ng/ml of LPS in the absence or presence of ginsenoside Rb1 (Rb1) or compound K (CK) (10 μM) for 30 min. (D) Effects on the interaction between LPS and TLR-4. Peritoneal macrophages isolated from mice were incubated with Alexa Fluor 488-conjugated LPS for 20 min in the absence (LPS) or presence of ginsenoside Rb1 (Rb1) or compound K (CK) (10 μM). (E) The effect on IRAK-1 phosphorylated degradation. They were determined by a confocal microscopy using an antibody for the IRAK-1 subunit. (F) Effect on IRAK-1 ubiquitination in LPS-stimulated peritoneal macrophages. Peritoneal macrophages were cultured in 6-well plates in RPMI 1640 with or without ginsenoside Rb1 or compound K for 1 h and were then treated with or without 50 ng/ml LPS for 30 min. Cell lysates supplemented with 9 ml of NET buffer were incubated overnight at 4 °C with 10 μL of IRAK-1 antibody, and incubated with 50 μL of protein A/G-Agarose was conducted for 1 h at 4 °C to precipitate immune complex. Samples were centrifuged and separated on SDS-PAGE and analyzed by immunoblot analysis. (G) Effects on IRAK-1 activation and p-IKK-β phosphorylation in siRNA-transfected peritoneal macrophages. They were detected by immunoblot analysis.

studies have shown that compound K has exhibited potent pharmacological effects, such as anti-inflammatory, anti-tumor and antidiabetic effects [15–17,28]. Nevertheless, their anti-inflammatory mechanism has not been clarified. Therefore, we evaluated the effects of compound K and ginsenoside Rb1 on TNBS-induced colitis and investigated their anti-inflammatory mechanism. When ginsenoside Rb1 and compound K were orally administered in TNBS-induced colitic mice, colitic symptoms, including colon shortening, macroscopic score, and MPO activity—an index of polymorphonuclear leukocyte accumulation, were improved. Ginsenoside Rb1 and compound K also inhibited the decrease in tight junction-associated protein expression. TNBS-induced inflammation was mediated by inflammatory mediators, including IL-1β, TNF-α, IL-6, nitric oxide, and prostaglandins, as previously reported [29]. Of these inflammatory mediators, pro-inflammatory cytokines, such as, TNF-α, IL-1β, and IL-6, may be activated through NF-κB, and also activate NF-κB [24,29]. Ginsenoside Rb1 and compound K inhibited the expressions of the proinflammatory cytokines IL-1β, TNF-α and IL-6 in TNBS-induced colitic mice, but increased IL-10, an anti-inflammatory cytokine. Furthermore, these ginsenosides also inhibited iNOS and COX-2 expressions and NF-κB activation. On the basis of these results, ginsenoside Rb1 and compound K may inhibit TNBS-induced colitis by regulating NF-κB activation.

Blood IL-1β and TNF-α levels are barely detectable in normal mice [30]. However, treatment with LPS increased serum IL-1β and TNF-α levels and caused inflammation, as previously reported [31]. In the present study, ginsenoside Rb1 and compound K inhibited the expressions of TNF-α and IL-1β, as well as COX-2 and iNOS, in LPS-stimulated peritoneal macrophages. Phosphorylated

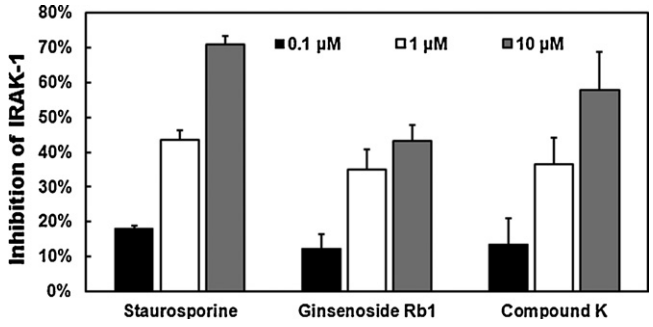

Fig. 9. Inhibitory effects of ginsenoside Rb1 or compound K on IRAK-1 kinase binding activity. To measure the inhibitory effects of ginsenoside Rb1 and compound K on IRAK-1 binding activity, the reaction mixture containing 5 nM IRAK-1, 2 nM Eu-Anti-GST Antibody, 100 nM Kinase Tracer 236[2] and a test compound (ginsenoside Rb1, compound K or staurosporine; 0.1, 1, 10 μM) in 1 × kinase Buffer A was incubated for 1 h at 25 °C. Fluorescence was measured by fluorescence multi plate reader.

IRAK-1 activates TAK1, which activates NF-κB via IκB kinase (IKK) complex and MAPKs [32]. We found that ginsenoside Rb1 and compound K inhibited LPS-induced IKK-β phosphorylation and the phosphorylation of the p65 subunit of NF-κB. Furthermore, they also inhibited the LPS-induced activation of the MAP kinases JNK, ERK, and p38. Of these MAP kinases, activation of p38 was most potently inhibited. On the basis of these results, ginsenoside Rb1 and compound K may regulate inflammatory markers by inhibiting LPS-induced IKK-β phosphorylation or its upstream signal transduction. Therefore, we investigated the roles of ginsenoside Rb1 and compound K in the interaction between LPS andTLR-4 and the activation of IRAKs. IRAK-1 is a key molecule in the signaling cascade of the Toll/IL-1 receptor family, although IRAK-2 continues to lead to NF-κB activation via IKKs phosphorylation in the absence of IRAK-1 [33]. IRAK-1 is activated via IRAK-4 after stimulants have been bound by TLRs, such as LPS/TLR-4. Activated IRAK-1 induces IKKs phosphorylation, which leads to the nuclear translocation of NF-κB. In the present study, ginsenoside Rb1 and compound K inhibited the formation of pUb-IRAK1 and the degradation of phosphorylated IRAK-1 in LPS-induced peritoneal macrophages. Therefore, ginsenoside Rb1 and compound K may inhibit the formation of K63-pUb-IRAK1, as staurosporine, which inhibits IRAK-1 phosphorylation [34,35]. Ginsenoside Rb1 and compound K also inhibited IRAK-1 kinase activity *in vitro*, as staurosporine, an IRAK-1 kinase inhibitor. However, IRAK-2 was not affected by ginsenoside Rb1 or compound K. Furthermore, ginsenoside Rb1 and compound K did not affect the interaction between LPS and its receptor, TLR-4,or the expression of IRAK-4, which leads to the phosphorylation of IRAK-1 on the cell membranes of peritoneal macrophages. However, neither ginsenoside Rb1 nor compound K reversed LPS-induced IKK-β phosphorylation in LPS-stimulated IRAK-1 siRNA-transfected peritoneal macrophages as was observed in macrophages not treated with LPS. Based on these findings, ginsenoside Rb1 and compound K may regulate IRAK-1 activation. The overexpressions of pro-inflammatory cytokines, such as, TNF-α and IL-1β, via NF-κB and MAPK pathways in macrophages have been observed in inflammatory diseases [36]. Ginsenoside Rb1 and compound K inhibited IRAK-1 activation, IKK-β phosphorylation, MAPK activation (JNK, ERK, and p38), and NF-κB activation in LPS-activated macrophages, but did not affect LPS/TLR-4 complex formation and IRAK-4 activation. Their inhibitory effects against MAPK and NF-κB activation may inhibit the expression of the proinflammatory cytokines TNF-α and IL-1β and of the inflammatory enzymes COX-2 and iNOS. Ginsenoside Rb1 and compound K may regulate MAPK and NF-κB pathways by inhibiting IRAK-1 phosphorylation.

Compound K inhibited the expressions of inflammatory markers (e.g., proinflammatory cytokines), IKK-β phosphorylation, and NF-κB activation, in LPS-treated peritoneal macrophages more potently than did ginsenoside Rb1. However, their anticolitic effects, such as colon length and proinflammatory cytokine expression, were not significantly different in vivo. On the basis of these results, orally administered ginsenoside Rb1 may be metabolized to compound K by intestinal microflora in the intestine, and ginsenoside Rb1 and its metabolite compound K inhibit inflammation in TNBS-induced colitic mice.

The current findings indicated that ginsenoside Rb1 and compound K may improve inflammatory diseases, such as colitis, by inhibiting IRAK-1 activation via TLR-4-linked NF-κB and MAPK pathways.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.bcp.2011.05.003.

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