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# Anti-inflammatory effects of ginsenoside Rg1 and its metabolites ginsenoside Rh1 and 20(S)-protopanaxatriol in mice with TNBS-induced colitis

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ABSTRACT

Ginsenoside Rg1, one of the main constituents of *Panax ginseng*, exhibits anti-inflammatory effect. In a preliminary study, it was observed that ginsenoside Rg1 was metabolized to 20(S)-protopanaxtriol via ginsenosides Rh1 and F1 by gut microbiota. We further investigated the anti-inflammatory effects of ginsenoside Rg1 and its metabolites in vitro and in vivo. Ginsenosides Rg1, Rh1, and 20(S)-protopanaxtriol inhibited the activation of NF-κB activation, phosphorylation of transforming growth factor beta-activated kinase 1 and interleukin (IL)-1 receptor-associated kinase, and expression of tumor necrosis factor-α and IL-1β in lipopolysaccharide (LPS)-stimulated macrophages. They also inhibited the binding of LPS to toll-like receptor 4 on the macrophages. Orally administered ginsenoside Rg1, Rh1, or 20(S)-protopanaxtriol inhibited 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colon shortening, myeloperoxidase activity, and expression of IL-1β, IL-17, and tumor necrosis factor-α in mice with TNBS-induced colitis. They did not only inhibit TNBS-induced NF-κB activation, but also restored TNBS-induced Th17/Treg imbalance. They restored IL-10 and Foxp3 expression. Moreover, they inhibited Th17 cell differentiation in vitro. Of these metabolites, in vitro and in vivo anti-inflammatory effect of 20(S)-protopanaxtriol was the most potent, followed by Rh1. These findings suggest that ginsenoside Rg1 is metabolized to 20(S)-protopanaxtriol via ginsenosides Rh1 and F1 and these metabolites particularly 20(S)-protopanaxtriol, may ameliorate inflammatory disease such as colitis by inhibiting the binding of LPS to TLR4 on macrophages and restoring the Th17/Treg imbalance.



## 1. Introduction

Inflammation is a highly regulated defense process in the body characterized by the release of inflammatory mediators and the transmigration of immune cells, such as neutrophils, monocytes, and lymphocytes, from the blood to the affected tissue (Johnson and Koval, 2009; Sotolongo et al., 2012; Perkins and Gilmore, 2006). Of these inflammatory mediators, pro-inflammatory cytokines tumor necrosis factor (TNF)-α and interleukin (IL)-1β are barely detectable in mice in the absence of stimuli such as pathogens or injury (Laveti et al., 2013; Fairweather and Rose, 2005). However, stimulation with lipopolysaccharide (LPS) or peptidoglycan can induce their expression by activating toll-like receptor (TLR)-NF-κB signaling pathways, leading to inflammation (Wahl et al., 2003; Chow et al., 1999; Olson and Miller, 2004).

TLRs connect the innate and adaptive mucosal immune responses. TLR4, a transmembrane co-receptor with CD14 in the cellular response to pathogens (Chow et al., 1999; Ingalls et al., 1999), serves as the primary mediator of LPS signaling. It then activates the transcription factor NF-κB via IL-1 receptor-associated kinases (IRAKs) and transforming growth factor beta-activated kinase (TAK)1 (Li and Verma, 2002; Sacre et al., 2007; Takeuchi and Akira, 2001). Therefore, regulating the expression of these inflammatory mediators can be beneficial in curing inflammatory disease.

Ginseng (the root of *Panax ginseng*, family Araliaceae), which contains protopanaxadiol-type ginsenosides Rb1 and Rb2 and protopanaxatriol-type ginsenosides Re and Rg1 as its main constituents, is used in the traditional Chinese medicine for inflammation, cancer, stress, and diabetes (Kim, 2012; Park et al.,

*Abbreviations:* compound K, 20-O-(β-D-glucopyranosyl)-20(S)-protopanaxadiol; COX, cyclooxygenase; ELISA, enzyme-linked immunosorbent assay; IL, interleukin; IRAK, IL-1 receptor-associated kinase; IκBα, nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor, alpha; iNOS, inducible NO synthase; LPS, lipopolysaccharide; TGF, transforming growth factor; TNF, tumor necrosis factor; TLR, toll-like receptor; TNBS, 2,4,6-trinitrobenzene sulfonic acid; TNF, tumor necrosis factor; TAK, transforming growth factor beta-activated kinase

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2012; Washida and Kitanaka, 2003). These ginsenosides exhibit anti-inflammatory effects (Kim, 2012; Park et al., 2005). Ginsenoside Rb1, which is metabolized to 20-O-(β-D-glucopyranosyl)-20 (S)-protopanaxadiol (compound K) by gut microbiota. Compound K inhibits LPS-stimulated inflammation by inhibiting IRAK1 in LPS-stimulated macrophages and ameliorates 2,4,6-trinitrobenzene sulfonic acid (TNBS)-induced colitis in mice (Joh et al., 2011). Of protopanaxatriol-type ginsenosides, the anti-colitic effect of ginsenoside Re was evaluated, and found to ameliorate TNBS-induced colitis by inhibiting the binding of LPS to TLR4 on macrophages (Lee et al., 2012). However, the anti-colitic effect of ginsenoside Rg1 has not been studied.

In a preliminary study, ginsenoside Rg1 was metabolized to 20 (S)-protopanaxatriol via ginsenoside Rh1 and ginsenoside F1 by the gut microbiota of humans and mice. We decided to further investigate their anti-inflammatory effects in LPS-induced macrophages, IL-6/tumor growth factor (TGF)β-induced Th17 cells, and mice with TNBS-induced colitis.

## 2. Materials and methods

### 2.1. Materials

TNBS, LPS purified from *Escherichia coli* O111:B4, peptidoglycan purified from *Streptococcus aureus,* and RPMI 1640 were purchased from Sigma-Aldrich (St. Louis, MO, USA). Antibodies for IRAK1, p-IRAK1, inducible NO synthase (iNOS), cyclooxygenase (COX)-2, and β-actin were purchased from Santa Cruz Biotechnology (Santa Cruz, L.A. USA). Antibodies for nuclear factor of kappa light polypeptide gene enhancer in B-cells inhibitor alpha (IκBα), p-IκBα, IκB kinase (IKK)β, p-IKKβ, TAK1, p-TAK1, p65 and p-p65 were purchased from Cell Signaling Technology (Beverly, MA, USA). Enzyme-linked immunosorbent assay (ELISA) kits for cytokines were purchased from R&D Systems (Minneapolis, MN, USA). Anti-CD3, anti-CD28, recombinant IL-6, and tumor growth factor (TGF)β were purchased from BioLegend Inc. (San Diego, CA, USA). Heat-inactivated fetal calf serum (FCS) and fetal bovine serum (FBS) were purchased from Invitrogen (Carlsbad, CA, USA). $CD4^+$ T Cell Isolation Kit II, anti-IL-17A, and anti-Foxp3 were purchased from MiltenyiBiotec (Bergisch Gladbach, Germany).

Ginsenoside Rg1 (purity $>95\%$), and its metabolites ginsenoside Rh1 (purity $>93\%$), and 20(S)-protopanaxatriol (purity $>93\%$) were isolated according to the previously published method of Bae et al. (2005).

### 2.2. Animals

Male ICR mice (20–23 g, 4.5 weeks old) were supplied from Orient Animal Breeding Center (Sungnam, Korea). All animals were fed standard laboratory chow, housed in wire cages at 20–22 °C and $50 \pm 10\%$ humidity, and allowed water *ad libitum*.

All experiments were performed in accordance with the NIH and Kyung Hee University guides for Laboratory Animals Care and Use and approved by the Committee for the Care and Use of Laboratory Animals in the College of Pharmacy, Kyung Hee University (IRB No. KHP2013-11-02-1).

### 2.3. Isolation and culture of peritoneal macrophages

Mice were intraperitoneally injected with 2 ml of 4% thioglycolate solution and killed 4 days after the injection. Their peritoneal cavities were swilled with 10 ml of RPMI 1640 and the peritoneal lavage fluids were collected and centrifuged (300*g*, 10 min) (Jang et al., 2014). Collected cells were resuspended with RPMI 1640 and plated. Cells were incubated at 37 °C and washed three times every 2 h. Nonadherent cells were removed by aspiration. Cells were cultured in 24-well plates ($0.5 \times 10^6$ cells/well) at 37 °C in RPMI 1640 with 10% FBS. Attached cells were used as peritoneal macrophages. To examine the anti-inflammatory effects of ginsenosides, macrophages were incubated in the absence or presence of ginseonsides with LPS or peptidoglycan.

### 2.4. Preparation of splenocytes

Spleens were aseptically removed from mice, then crushed into a single cell suspension, and lysed with Tris-buffered ammonium chloride (Okamoto et al., 2012). The cell suspension was prepared in RPMI 1640 medium containing 10% FCS. $CD4^+$ T cells were isolated from splenocytes by Pan T Cell Isolation Kit II. The purified Pan T cells were stimulated with anti-CD3 (1 μg/ml) and anti-CD28 (1 μg/ml) in the presence of recombinant IL-6 (20 ng/ml) and recombinant TGFβ (1 ng/ml) with or without ginsenosides for 4 days.

### 2.5. ELISA and immunoblot analysis in peritoneal macrophages

Cells ($0.5 \times 10^6$ cells) were stimulated with LPS (100 ng/ml) for 90 min (immunoblotting except iNOS and COX2) or 20 h (for ELISA and immunoblotting of iNOS and COX2) in the absence or presence of ginseonsides (10 and 20 μM), lysed, and centrifuged (2000*g*, 10 min). For the ELISA assay, cell supernatents prepared from macrophages were transferred to 96-well ELISA plates. Cytokines levels were determined using ELISA kits (Jang et al., 2014). For the immunoblot analysis, cell supernatants were applied to 10% SDS-PAGE and transferred onto polyvinylidene difluoride membranes (Jang et al., 2014). The membranes were blocked with 5% non-fat dried-milk proteins in 0.05% phosphate-buffered saline with tween 20 (PBST), then probed with COX-2, iNOS, IRAK1, p-IRAK1, TAK1, p-TAK1, p-IκBα, IκBα, p65, p-p6, or β-actin antibody. After washing with PBST, proteins were detected with horseradish peroxidase (HRP)-conjugated secondary antibodies for 50 min. Bands were visualized with enhanced chemiluminescence detection kit.

### 2.6. Preparation of experimental colitis in mice

To investigate the curative effect of ginsenoside Rg1 and its metabolites Rh1 and 20(S)-protopanaxatriol against colitis in mice, the mice were divided into seven groups: normal and TNBS-induced colitic groups treated with or without ginsenoside Rg1, Rh1 (20 mg/kg), 20(S)-protopanaxatriol (10 and 20 mg/kg), or sulfasalazine (50 mg/kg). Colitis was induced by the intra-rectal injection of 2.5% (w/v) TNBS solution (100 μL) in 50% ethanol into the colon of lightly anesthetized mice via a round-tip needle equipped with a syringe (Jang et al., 2014). Normal group was treated with vehicle alone. TNBS–ethanol solution quickly excreted mice were excluded: $>95\%$ of the mice caused TNBS enema. Test agents were orally administered once a day for 3 days from the 1st day after TNBS treatment. Mice were killed 18 h after the final administration of test agents. Colon was quickly removed, opened longitudinally, and gently washed with ice-cold phosphate buffered saline. Macroscopic assessment of the colitis grade was scored according to a previously reported scoring system (Jang et al., 2014) and the colon tissue was then stored at −80 °C until the experiment.

For the histological exam, the colons were fixed in 10%-buffered formalin solution, cut into 10-μm sections, stained with hematoxylin–eosin, toluidine blue, or safranin O, and observed under a light microscopy.