Arch Pharm Res Vol 29, No 8, 685-690, 2006

Archives of Pharmacal Research
http://apr.psk.or.kr

Inhibitory Effect of Ginsenoside Rg5 and Its Metabolite Ginsenoside Rh3 in an Oxazolone-Induced Mouse Chronic Dermatitis Model

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(Received May 12, 2006)

The effect of a main constituent ginsenoside Rg5 isolated from red ginseng and its metabolite ginsenoside Rh3 in a chronic dermatitis model was investigated. Ginsenosides Rg5 and Rh3 suppressed swelling of oxazolone-induced mouse ear contact dermatitis. These ginsenosides also reduced mRNA expressions of cyclooxygenase-2, interleukin (IL)-1β, tumor necrosis factor (TNF)-α and interferon (IFN)-γ. The inhibition of ginsenoside Rh3 was more potent than that of ginsenoside Rg5. These findings suggest that ginsenoside Rh3 metabolized from ginsenoside Rg5 may improve chronic dermatitis or psoriasis by the regulation of IL-1β and TNF-α produced by macrophage cells and of IFN-γ produced by Th cells.

Key words: Ginsenoside Rg5, Ginsenoside Rh3, COX-2, IFN-γ, Psoriasis

INTRODUCTION

Psoriasis is a chronic dermatitis. Psoriasis patients have been shown to have interferon (IFN)-γ producing Th1 bias in lesion skin and peripheral blood, although cylcooxygenase (COX)-2 is also induced, and to develop cytokine net works of Th1 cells (Austin *et al.*, 1999; Nicoloff, 1991; Hernandez *et al.*, 2001). Fujii *et al.* (2002) developed oxazolone-induced mouse contact dermatitis as an experimental psoriatic animal. Ginseng (the root of *Panax ginseng* C.A. Meyer, Araliaceae) is frequently taken orally as a traditional medicine in Asian countries. The main components of raw ginseng are ginsenosides Rb1, Rb2, Rc and Rf. (Shibata *et al.*, 1963). However, those of red ginseng or heat-processed ginseng are ginsenosides Rg3 and Rg5 transformed from protopanaxadiol ginsenosides by the heating process to prepare them (Kitagawa *et al.*, 1983; Kown *et al.*, 2001). These ginsenosides have been reported to exhibit various biological activities, including anti-allergic (Choo *et al.*, 2003; Park *et al.*, 2004), anti-inflammatory action (Wu *et al.*, 1992), and anti-tumor effects (Mochizuki *et al.*, 1995; Wakabayashi et al., 1998). The pharmacological actions of these ginsenosides are activated by human intestinal microflora (Wakabayashi *et al.*, 1998; Akao *et al.*, 1998). For example, ginsenosides Rg3 is metabolized to ginsenoside Rh2 by human intestinal microflora (Bae *et al.*, 2003). The metabolized ginsenoside Rh2 exhibited more potent anti-allergic effects such as anti-passive cutaneous anaphylaxis and anti-inflammatory effects such as the inhibition of prostaglandin E2 biosynthesis, than ginsenoside Rg3 (Park *et al.*, 2003, 2004). However, effect of ginsenoside Rg5 and its metabolite ginsenoside Rh3 against chronic contact dermatitis has not been studied.

Therefore, inhibitory effect of ginsenoside Rg5 and its metabolite ginsenoside Rh3 in oxazolone-induced mouse ear contact dermatitis was investigated.

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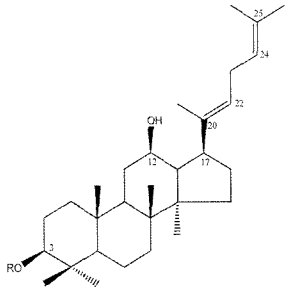
MATERIALS AND METHODS

Materials

Oxazolone and betamethasone were purchased from Sigma Co. (St Louis, MO, U.S.A). Ginsenosides Rg5 and Rh3 (Fig. 1) were isolated according to the previously reported method (Kwon *et al.*, 2001; Kim *et al.*, 1995).

Animals

The female ICR mice (20-25 g) were supplied from Orient Experimental Animal Breeding Center (Seoul,



	R
Ginsenoside Rg5	Glc-Glc-
Ginsenoside Rh3	Glc-

Fig. 1. Structure of Ginsenosides Rg5 and Rh3

Korea). All animals were housed in wire cages at 20-22°C and 50 ± 10% humidity, fed standard laboratory chow (Orient Experimental Animal Breeding Center, 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).

Oxazolone-induced dermatitis

An oxazolone-induced dermatitis was measured according to the previous method of Fujii *et al.* (2002). Each group contained 5 female ICR mice (20-25 g). Mice were sensitized by application of 100 μL of 1.5% oxazolone in ethanol to the abdomen. Then a total of 20 μL of 1% oxazolone in a mixture of acetone and olive oil (4:1) was applied to both sides of the mouse ear every 3 days starting from 7 days after sensitization. Ear thickness was measured using a Digimatic Micrometer (Mitsutoyo Co., Tokyo, Japan) 72 h after each application of the oxazolone, test agents (0.02% or 0.05%) were applied in a total volume of 20 μL to both sides of the ear 30 min before and 3 h after each application of oxazolone

Histopathological study

Mouse ears were excised 72 h after the last application of oxazolone and fixed in 10%-buffered formalin solution, embedded in paraffin by standard methods, cut into 5-mm sections, stained with hematoxylin-eosin, and then assessed under light microscopy.

Reverse transcription-polymerase chain reaction (RT-PCR) analysis

Ear tissue extract for RT-PCR analysis was performed by the method of Shin *et al.* (2005). Briefly, ears were excised 6 h after the last application of oxazolone, freezed in liquid nitrogen and homogenized by a mortar and pestle prechilled in liquid nitrogen. Total RNA was extracted by using TRI reagent according to the manufacturer's instructions, and treated with RNase-free DNase. The concentration of RNA content was determined by measuring the absorbance at 260 and 280 nm and stored at -70°C until RT-PCR analysis. The RT-PCR was performed with AccPower® RT/PCR Premix (Bioneer, Seoul, Korea). The primers were designed as described by UniSTS database: COX-1, forward primer 5'-CTTTTATCCTCCCAGGATTTGG -3 and reverse primer 5'-GCTAAATACTTTGACACCGG-3' (product size 231 bp); COX-2 (UniSTS 254306), forward primer 5'-TGTATCCCCCCACAGTCAAAGACAC-3 and reverse primer 5'-GTGCTCCCGAAGCCAGATGG-3' (product size 146 bp); IL-1β, forward primer 5'- ATGGCAACT-GTCCCTGAACT-3 and reverse primer 5'- GTCGTTGCT-TGTCTCTCCTT-3' (product size 508 bp); IFN-γ (UniSTS 160031), forward primer 5'-CTTTAACAGCAGGCCAGACA-3' and reverse primer 5'-GCGAGTTATTTGTCATTCGG-3' (product size 144 bp); IL-4 (UniSTS 143568), forward primer 5'-CCGATTATGGTGTAATTTCCTATGCTG-3' and reverse primer 5'GGCCAATCAGCACCTCTCTTCCAG-3' (product size 111 bp); tumor necrosis factor (TNF)-α (UniSTS 209165), forward primer 5'-GATTTTATTTGTTTA-AAAGCAGATATC-3' and reverse primer 5'-CATCCTAAG-TCTACACAGGATCT-3' (product size 206 bp); GAPDH (UniSTS 225899), forward primer 5'-ACCACAGTCCAT-GCCATCAC-3' and reverse primer 5'-TCCACCACCCTG-TTGCTGTA-3' (product size 452 bp). The RT-PCR products were electrophoresed on 2% agarose gel in TBE buffer, stained with ethidium bromide and photographed under UV light. The GAPDH gene was used as an internal control. The signal intensity of each RT-PCR product was estimated by Shimazu 9301-PC scanner (Tokyo, Japan).

Nuclear extract preparation and electrophoretic mobility shift assay (EMSA)

To prepare nuclear extract, each mouse ear was freezed and homogenized with Pellet Pestle Cordless Drive Unit (Thomas Scientific, Swedesboro, NJ, U.S.A.) and suspended in 1 mL of lysis buffer (10 mM Tris-HCl, pH 7.9, 10 mM NaCl, 3 mM $MgCl_2$, 1% NP-40) on ice for 4 min. After 10 min of centrifugation at 3,000 rpm, the pellet was resuspended in 50 μL of extraction buffer (20 mM HEPES, pH 7.9, 20% glycerol, 1.5 mM $MgCl_2$, 0.2 mM EDTA, 1 mM dithiothreitol, 1mM phenylmethylsulfonyl fluoride) and incubated on ice for 30 min. After centrifugation at 15,000 ×g for 5 min, the supernatant was harvested as the nuclear protein extract and stored at -70°C. Its protein concentration was determined with a protein assay reagent. Five micrograms of the nuclear proteins were incubated with ^{32}P-labeled NF-κB probe on ice for 30 min and resolved on a 5% acrylamide gel as previously described. For the supershift assay, antibodies against p65 or p50 subunits of NF-κB (Santa Cruz, CA) were co-incubated with the protein in the reaction mixture for 30 min at 4°C

prior to adding the radiolabeled probe.

Statistical analysis

All the data were expressed as mean ± standard deviation, and statistical significance was analyzed by one way ANOVA followed by Student-Newman-Keuls test.

RESULTS AND DISCUSSION

The effect of ginsenoside Rg5 and its metabolite ginsenoside Rh3 in oxazolone-induced mouse ear chronic dermatitis was evaluated (Fig. 2). The ear applied with oxazolone to sensitized mice caused erythema (reddening of the skin), edema and/or induration, and sometimes abrasion. The ear thickness as an index of skin inflammation measured 16th day after sensitization increased about 3.2-fold, compared with that of normal control. Betamethasone used as a positive agent at a dose of 0.05% potently suppressed ear swelling with a suppressive rate of 73.2% at 16th day. Ginsenosides Rg5 and Rh3 suppressed ear swelling at each time-point and their suppressive rates at a dose of 0.05% were 26.9% and 34.1% at 16th day,

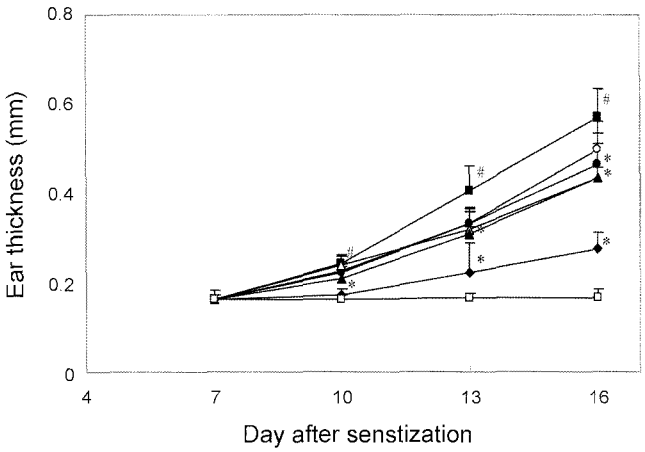


Fig. 2. Effects of Ginsenosides Rg5 and Rh3 on the Ear Thickness of Mice Induced by Oxazolone. ●, oxazolone alone treated control; ○, vehicle alone (negative normal) control; △, 0.02% ginsenoside Rg5 with oxazolone; ▲, 0.05% ginsenoside Rg5 with oxazolone; □, 0.02% ginsenoside Rh3 with oxazolone; ■, 0.05% ginsenoside Rh3 with oxazolone; ◆, 0.05% betamethasone. Values represent means ± S.D. for five mice. #Significantly different from the normal control group (#P<0.05). *Significantly different from the control group (*P<0.05).

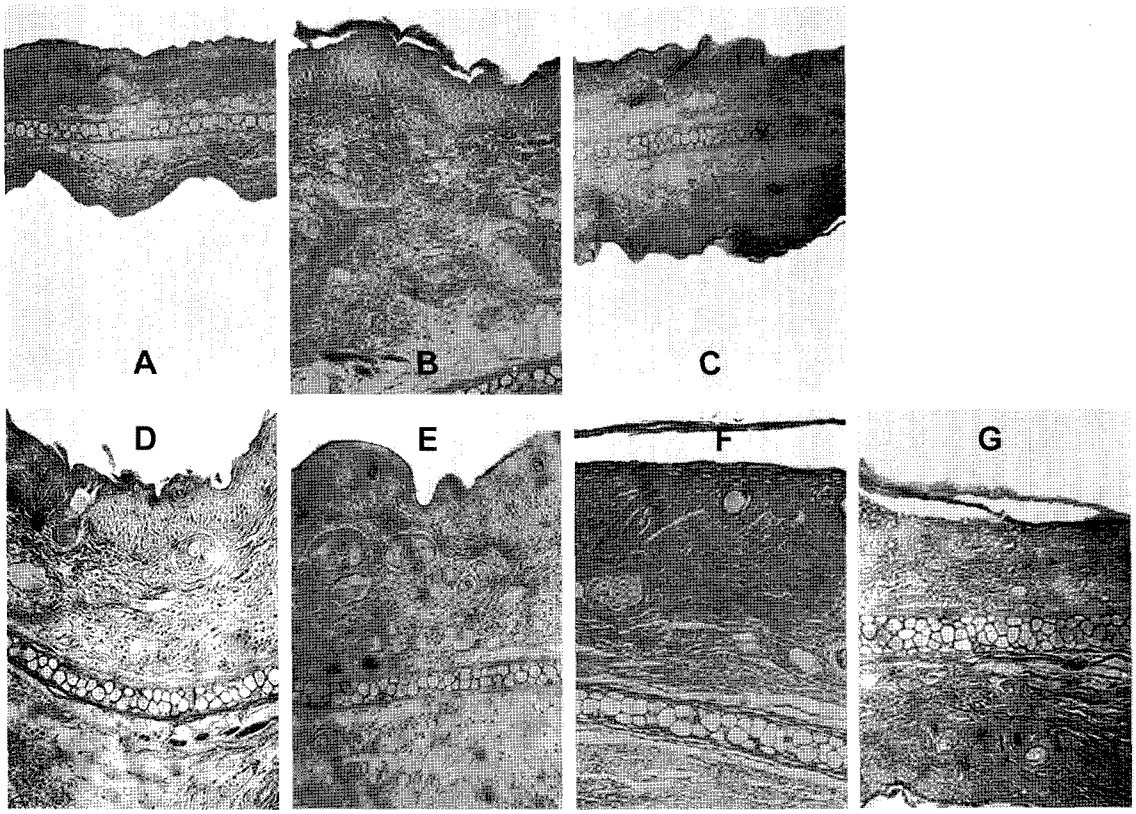


Fig. 3. Histopathological pictures of mouse ear after repeated application of oxazolone with or without ginsenosides. Mouse ears were excised 72 h after the last application of oxazolone and stained with hematoxylin-eosin. As a negative control, mice were only sensitized with 1.5% oxazolone to obdomen followed by no application to the ear (A). 1 % Oxazolone was applied to both sides of the ear every 3 days starting from 7 days after sensitization, and vehicle (ethanol) (B). 0.05% Betamethasone (C), 0.02% ginsenoside Rg5 (D), 0.05% ginsenoside Rg5 (E), 0.02% ginsenoside Rh3 (F), or 0.05% ginsenoside Rh3 (G) was applied to the ear 30 min before and 3 h after each application of oxazolone.

respectively. The inhibitory activity of ginsenoside Rh3 was more potent than that of ginsenoside Rg5.

For histopathological analysis, we excised the ear at 16 days and stained it with hematoxylin-eosin (Fig. 3). The ear applied with oxazolone swelled so dramatically that the entire section could not be shown. Betamethasone used as a positive agent at concentration of 0.05% potently suppressed ear swelling with a suppressive rate of 73.2% at 16th day. Ginsenosides Rg5 and Rh3 also potently suppressed ear swelling at each time-point. The ginsenoside Rh3 inhibited the increment of epidermal thickness of ear treated by oxazolone more potently than ginsenside Rg5. In histopathological analysis by staining with hematoxylin-eosin, the ginsenoside Rh3 recovered the injured ear that the entire section could not be shown by the application with oxazolone.

The effect of ginsenosides Rg5 and Rh3 in mRNA expression levels of COX-1, COX-2 and some cytokines of mouse ear chronic dermatitis induced by oxazolone was investigated by using RT-PCR analysis (Fig. 4A). Oxazolone significantly induced mRNA expression level of COX-2, but did not induce that of COX-1. The application of ginsenosides Rg5 and Rh3 in oxazolone-stimulated mouse inhibited mRNA levels of COX-2. These ginsenosides also inhibited mRNA levels of TNF-α and IL-1β increased by oxazolone. The ginsenoside Rh3 also inhibited IFN-γ mRNA expression, which are induced by Th1 cells. The ginsenoside Rh3 more potently inhibited mRNA expression of COX-2, TNF-α, IL-1β and IFN-γ than ginsenoside Rg5. However, the ginsenosides almost did not affect mRNA expression of IL-4 and COX-1.

To further dissect the anti-dermatitis mechanism of ginsenosides Rg5 and Rh3, effect of these agents on a transcription factor NF-κB, which modulates inflammatory reactions, was examined. As shown in Fig. 4B, elecrophoretic mobility shift assay (EMSA) revealed that ginsenosides Rg5 and Rh3 significantly repressed NF-κB DNA binding activity in oxazolone-induced mouse ear skins.

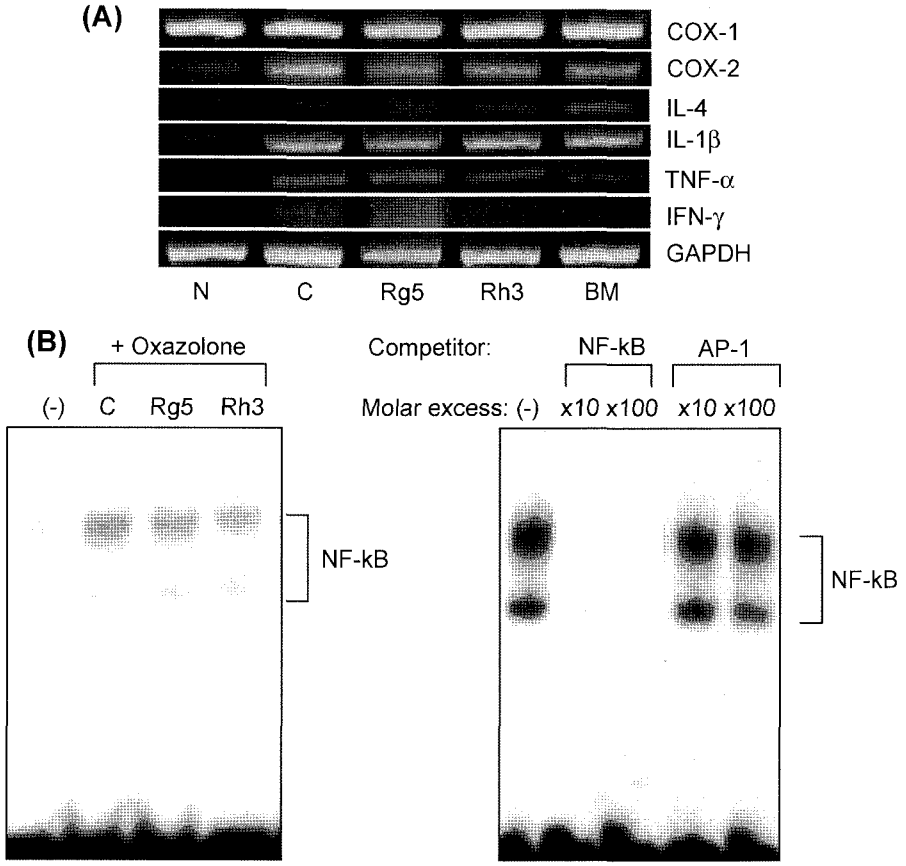


Fig. 4. Effect of ginsenosides Rg5 and Rh3 on mRNA expression of some enzymes and cytokines in oxazolone-induced mouse ear dermatitis. (A) effect of ginsenosides on mRNA expression levels of COX-1, COX-2, IL-1β, IL-4, INF-γ, TNF-α and GAPDH. N, Normal; C, oxazolone alone treated; Rg5, 0.05% ginsenoside Rg5 treated; Rh3, 0.05% ginsenoside Rh3 treated; BM, betamethasone treated. (B) Effect of ginsenosides on NF-κB activity. EMSA for NF-κB DNA binding activity. Nuclear extracts were prepared from mouse ear skins after treatment with oxazolone for 6 h in the presence or absence of indicated concentrations of ginsenosides Rg5 or Rh3. NF-κB-DNA complex is indicated as an arrow. The DNA-protein complex indicated by the arrow was competed by cold NF-κB, but not by AP-1 oligonucleotide, indicating that the complex is NF-κB sequence-specific.

Steroids, antihistamines and immunosuppressants have dermatitis or psoriasis (Schafer-Korting *et al.*, 1996; Sakuma *et al.*, 2001; Simons *et al.*, 1992). Nevertheless, the topical use of these corticosteroids can cause intense skin atrophy, one of the serious side effects limiting their uses for chronic skin diseases. Therefore, new agents for clinical uses have been developed from Chinese traditional medicines (Bielory, 2004).

Ginseng (the root of *Panax ginseng* C.A. Meyer, family Araliaceae) was found to show inhibitory activity in an oxazolone-induced mouse ear chronic dermatitis model. Its protopanaxadiol ginsenoside metabolite compound K showed the potent inhibition against dermatitis models (Shin *et al.*, 2005). However, the effect of ginsenoside Rg5 isolated from red ginseng and its metabolite ginsenoside Rh3 against chronic dermatitis and their inhibitory mechanism have not been studied.

Oxazolone-induced dermatitis prepared in the present study was accompanied by substained swelling and predominant epidermal hyperplasia as reported by Fujii *et al.* (2002). IFN-γ, IL-1β and TNF-α, which are cytokines involved in chronic skin inflammatory disease (Austin *et al.*, 1999; Nicoloff, 1991), and COX-2, which is a marker of inflammatory diseases (Hernandez *et al.*, 2001), were also induced in oxazolone-induced mouse ear dermatitis. Ginsenosides Rg5 and Rh3 significantly inhibit sustained swelling (thickness) of mouse ears induced by oxazolone. These ginsenosides not only inhibited the mRNA expression of COX-2, but also the activation of NF-κB transcription factor, which is an upstream modulator of COX-2 gene expression in mouse ears (Hernandez *et al.*, 2001; Keum *et al.*, 2003). However, protopanxadiol ginsenosides compound K and ginsenoside Rh2 did not inhibit commercial COX-2 activity (Shin *et al.*, 2005), although ginsenosides Rg5 and Rh3 were not measured. These findingss suggest that contact dermatitis-inhibitory mechanism of these ginsenosides may be regulated by the activation of transcripition factor NF-κB in macrophages and monocytes (Keum *et al.*, 2003). Furthermore, the ginsenosides Rg5 and Rh3 significantly inhibited mRNA expression of TNF-α and IL-1β produced by macrophages, and IFN-γ produced by Th1 cells, although it weakly inhibited that of IL-4 produced by Th2 cells. These results suggest that ginsenosides Rg5 and Rh3 can improve inflammatory skin disorders, such as contact dermatitis or psoriasis, by the regulation of COX-2, TNF-α, and IL-1β produced by macrophage cells and IFN-γ produced by Th1 cells. The inhibitory effect of ginsenoside Rh3 was more potent than that of ginsenoside Rg5. Nevertheless, the effect of orally admininstered ginsenoside Rg5 may be similar to that of ginsenoside Rh3. Because orally administered ginsenoside Rg5 can be easily metabolized to ginsenoside Rh3 by intestinal microflora (Bae *et al.*, 2002). Based on these findings, we believe that ginsenoside Rg5 may be metabolized to ginsenoside Rh3 by intestinal microflora and the metabolized ginsneoside Rh3 can improve inflammatory skin disorders, chronic dermatitis or psoriasis.

ACKNOWLEDGEMENTS

This work was supported by a grant from the Korea Science and Engineering Foundation (No. R01-2002-00220). We would like to thank Prof. Hee Sun Kim, School of Medicine, Ewha University, for participating in EMSA assay.

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