Inhibitory Effect of Ginsenoside Rb1 and Compound K on NO and Prostaglandin E2 Biosyntheses of RAW264.7 Cells Induced by Lipopolysaccharide

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Received August 25, 2004; accepted December 2, 2004

In this study, the antiinflammatory activities of ginsenoside Rb1, which is a main constituent of the root of Panax ginseng (Araliaceae), and of its metabolite compound K, as produced by human intestinal bacteria, on lipopolysaccharide (LPS)-induced RAW264.7 cells were investigated. Compound K potently inhibited the production of NO and prostaglandin E2 in LPS-induced RAW 264.7 cells, with IC50 values of 0.012 and 0.004 mM, respectively. Compound K also reduced the expression levels of the inducible NO synthase (iNOS) and COX-2 proteins and inhibited the activation of NF-kB, a nuclear transcription factor. Compound K inhibited the NO level produced by iNOS enzyme activity in a cell-free system, but did not inhibit COX-1 and 2 activities. When ginsenoside Rb1 was orally administered to rats, compound K, but not ginsenoside Rb1, were excreted in their urine. These findings suggest that ginsenoside Rb1 can be transformed to compound K by intestinal bacteria, and compound K may be effective against inflammation.

Key words ginseng; compound K; COX-2; inducible NO synthase (iNOS); inflammation

Ginseng (the root of Panax ginseng C.A. MEYER, Araliaceae) is frequently taken orally, as a crude substance, as a traditional medicine in Asian countries. The major components of ginseng are ginsenosides, which contain an aglycone with a dammarane skeleton.1,2) These ginsenosides have been reported to exhibit various biological activities, including anti-inflammatory action and antitumor effects (inhibition of tumor-induced angiogenesis and the prevention of tumor invasion and metastasis).3—5) The pharmacological actions of these ginsenosides have been explained by their biotransformation by human intestinal bacteria.5—8) For example, protopanaxadiol ginsenosides are transformed to 20-\(\beta\)-D-glucopyranosyl-20(\(S\))-protopanaxadiol (compound K) by human intestinal bacteria. The metabolite compound K induces an antimetastatic or anticarcinogenic effect by blocking tumor invasion or preventing chromosomal aberration and tumorigenesis.5) Ginsenosides Re and Rg1 are also transformed to ginsenoside Rh1 or 20(\(S\))-protopanaxatriol, which have exhibited potent antiallergic and antiinflammatory effects.9—12)

However, the antiinflammatory effect of protopanaxadiol ginsenosides, such as ginsenoside Rb1, and compound K, has not been studied.

Therefore, we isolated ginsenoside Rb1 from ginseng and its metabolite compound K and investigated the antiinflammatory effect of ginsenoside Rb1 and its metabolite compound K (Fig. 1), using RAW264.7 cell induced by lipopolysaccharide (LPS).

MATERIALS AND METHODS

Materials \(N^G\)-monomethyl-L-arginine (\(L\)-NMMA), lipopolysaccharide (LPS), dexamethasone and indomethacin were purchased from Sigma Chemical Co. (St Louis, MO, U.S.A.). The Griess reagent was purchased from Promega Co. (Madison, WI, U.S.A.). The anti-rabbit inducible NO synthase (iNOS), anti-goat COX-2, anti-rabbit nuclear factor-kB (NF-kB) and anti-mouse \(\beta\)-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). The prostaglandin E2 (PGE2) EIA kit and COX (ovine) Inhibitor Screening Assay kits were purchased from Cayman Chemical (Ann Arbor, MI, U.S.A.). The enhanced chemiluminescence (ECL) western blot system was from Pierce Co. (Rockford, IL, U.S.A.).

The RAW 264.7 cells were purchased from the Korean Cell Line Bank (Seoul, Korea).

Isolation of Ginsenoside Rb1 and Its Metabolite Compound K by Human Intestinal Microflora Ginsenoside Rb1 and compound K were isolated from fermented Ginseng according to the previously published method.2,13) Ginsenoside Rb1 (2 g) from a BuOH extract of white ginseng (Kyung Dong Market, Seoul, Korea), was isolated by silica gel column chromatography using \(\text{CHCl}_3–\text{MeOH–H}_2\text{O} (10 : 3 : 1, \text{lower layer})\), according to the previously reported methods.13) Fresh human feces (5 g) were suspended in TS broth, centrifuged at 500\(\times g\) for 10 min, and the resulting supernatant

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Fig. 1. Structure of Ginsenoside Rb1 and Compound K
was centrifuged at 10000×g for 30 min, then washed twice with TS broth. The resulting precipitate was washed in 100 ml water containing 1% ginsenoside Rb1, and incubated for 24 h at 37 °C, then extracted with BuOH. This BuOH fraction was chromatographed on a silica gel column using CHCl3–MeOH–H2O (10:3:1, lower layer) to isolate compound K (120 mg). The isolated compound K was recrystallized with EtOH. Its purity was assayed by HPLC (Hitachi HPLC system: column, Lichrosorb NH2 (25×0.4 cm, 5 mm, Merck Co.); elution solvent, mixtures of solvent acetonitrile/water/isopropanol=80:5:15 and solvent B (acetonitrile/water/isopropanol=80:20:15)-gradient profile of solvent A to solvent B from 70:30 to 0:100 for 0—20 min and from 0:100 for 20—60 min; Detector, ELSD 800 (Alltech Associates, Inc., Deerfield, IL, U.S.A.).

Ginsenoside Rb1 (purity, >90%): White powder, mp 197—198 °C (dec.) FAB-MS (m/z) 1110 [M+1]+.

Compound K (purity, >97%): Colorless needles, mp 220—221 °C (dec.) EI-MS (m/z) 623 [M]+.

**Measurement of NO Production in LPS-Induced RAW264.7 Cells** Measurement of NO production in RAW264.7 cells (5×104 cells/well) was performed according to the method of Ishihara et al.15) The RAW 264.7 cells were plated in 60 mm culture dishes (3×104 cells), test agents and LPS (1 µg/ml) were added to the culture medium, and the cells were incubated at 37 °C for 6 h (in NF-kB) or 20 h (in iNOS and COX-2). The cells were lysed on ice for 15 min in a hypotonic buffer containing 10 mM Tris (pH 8.0), 1.5 mM MgCl2, 1 mM DTT, 0.1% NP-40, 5 µg/ml pepstatin A and 5 µg/ml aprotinin, and centrifuged at 12000×g at 4 °C for 15 min. Supernatant was used as the cytosol fraction for the immunoblot assays for the iNOS and COX-2 proteins. The precipitated nuclei fractions for the immunoblot assays of the NF-kB protein expression were resuspended in an extraction buffer, which contained 10 mM Tris (pH 8.0), 50 mM KCl, 300 mM NaCl, 1 mM DTT, 5 µg/ml pepstatin A and 5 µg/ml aprotinin. The mixture was then lysed on ice for 30 min. The lysed nuclei fraction was centrifuged at 12000×g at 4 °C for 30 min. The resulting supernatant was used as a nuclei fraction for the analysis of NF-kB protein expression level. Protein expression levels of COX-2, iNOS, NF-kB and β-actin of the cell lysates (40 µg) were analyzed by the above immunoblot method.

**Pharmacokinetics Study** Ginsenoside Rb1 (50 mg/rat) was orally administered to three male SD rats (250—260 g), and urine was collected periodically at 3 h, 6 h, 12 and 24 h postdosing. The urine samples were treated according to the previously reported method. The urine (3 ml) was incubated at 37 °C for 20 h with β-glucuronidase (10000 units, Sigma Co. MO, U.S.A.) in 1 ml of 1 M sodium acetate buffer (pH 4.5), and then applied on a preconditioned C18 solid-phase column (Sep-Pak, Waters Co., U.S.A.) preconditioned with 6 ml of 95% ethanol and 10 ml of distilled water. The column was washed with 4 ml of 10% methanol in distilled water and then eluted with 2 ml of methanol. The eluted sample was analyzed by HPLC [Hitachi System: column, µ-Bondapak C18 (3.9×300 mm); elution solvent, methanol/water/acetonitrile (20:30:50); elution rate, 1.0 ml/min; detector, ELSD 800]. Ginsenoside Rg3 was used as an internal standard. The retention times of ginsenoside Rb1 and compound K were as follows: ginsenoside Rb1, 3.24 min; ginsenoside Rg3 (internal standard), 5.02 min; and compound K, 8.58 min.

**Statistical Analysis** All the data from the experiment were expressed as mean±standard deviation and the statistical significance was determined using Student’s t-test.

**RESULTS** To investigate the antiinflammatory effect of ginsenoside...
Rb1 and compound K on RAW264.7 cells, we examined whether ginsenoside Rb1 and compound K could modulate NO synthesis in LPS-stimulated cultures of the murine macrophages of the RAW 264.7 cells (Table 1). The compound K significantly inhibited the production of NO in LPS-induced RAW 264.7 cells. Its IC50 was 0.032 mM. To see the direct inhibition of the iNOS enzyme activity, NO production produced by the enzyme activity was measured with or without compound K in the cell-free system (Fig. 2). When the RAW 264.7 cells were treated with LPS, NO level produced by the iNOS enzyme activity was significantly increased. The treatment of compound K at a concentration of more than 5 mM inhibited NO production. However, ginsenoside Rb1 did not inhibit the NO production in intact cell and cell-free systems.

Stimulation of the RAW 264.7 cells with LPS also increased the synthesis of PGE2, as shown in Table 1. Compound K significantly inhibited the production of NO in LPS-induced RAW 264.7 cells. Its IC50 was 0.032 mM. To see the direct inhibition of the iNOS enzyme activity, NO production produced by the enzyme activity was measured with or without compound K in the cell-free system (Fig. 2). When the RAW 264.7 cells were treated with LPS, NO level produced by the iNOS enzyme activity was significantly increased. The treatment of compound K at a concentration of more than 5 mM inhibited NO production. However, ginsenoside Rb1 did not inhibit the NO production in intact cell and cell-free systems.

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Choo et al. reported that compound K inhibited potently passive cutaneous anaphylaxis reaction in mice, but ginsenoside Rb1 did not. Therefore, to compare the biological activity of these ginsenosides and evaluate their antiinflammatory effect against allergic inflammation, we investigated the antiinflammatory activity of compound K in LPS-induced RAW264.7 cells. Compound K inhibited the production of NO and PGE2 in LPS-induced RAW 264.7 cells. Compound K inhibited NO production in a cell-free system. However, compound K did not inhibit the COX-1 and COX-2 enzyme activities. This result suggests that compound K may contribute to the iNOS enzyme activity.

Compound K inhibited the expression of the iNOS and COX-2 proteins, although the reason compound K at a concentration of 25 μM induced COX-2 protein level could not be understood. Compound K also inhibited the activation of the NF-κB transcription factor, which regulates iNOS and COX-2 gene expressions on LPS-induced RAW264.7 cells. These results suggest that compound K can inhibit NO and PGE2 production by regulation of the signal transduction related to the activation of NF-κB.

In addition, we previously reported that compound K may be responsible for passive cutaneous anaphylaxis reaction inhibitory activities, such as atopic diseases, and that protopanaxadiol ginsenosides of ginseng were easily metabolized to compound K, when ginseng extract or ginsenoside was orally administered. In the present study, compound K, but not ginsenoside Rb1, was detected in urine when ginsenoside Rb1 was orally administered to rats (Fig. 5). However, the amount of compound K recovered in the urine was 0.3% of the orally administered ginsenoside Rb1. The residual ginsenoside was not only absorbed into the blood, but also slowly excreted in the urine. Based on these findings, ginsenoside Rb1 may be transformed to compound K, which can be effective for treating allergic inflammation.

**Acknowledgements** This work was supported by a grant from the Korean Food Research Institute (2003).

**REFERENCES**