Ginsenoside Rh2 Reduces Ischemic Brain Injury in Rats

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Ginseng was incubated under mildly acidic conditions and its inhibitory effect on a rat ischemia-reperfusion model was investigated. When ginseng was treated with 0.1% hydrochloric acid at 60 °C, its protopanaxadiol saponins were transformed to diasteromeric ginsenoside Rg3 and \( \Delta^{20} \)-ginsenoside Rg3. When the transformed ginseng extract, of which the main component was ginsenosides Rg3, was treated with human intestinal microflora, the main metabolite was ginsenoside Rh2. Orally administered acid-treated ginseng (AG) extract and ginsenoside Rh2 potently protect ischemia-reperfusion brain injury. The ginsenoside Rh2 also inhibited prostaglandin-E\(_2\) synthesis in lipopolysaccharide-stimulated RAW264.7 cells, but showed no in vitro antioxidant activity. These results suggest that AG and ginsenoside Rh2 can improve ischemic brain injury.

Key words  ginseng; intestinal bacteria; ginsenoside Rh2; ischemia; prostaglandin-E\(_2\)

An inflammatory reaction in the brain, due to intervention or decrease in blood circulation, causes an ischemic stroke, and occurs in about 80% of brain stroke patients. The damage to brain neuronal cells due to an inflammatory reaction causes the release of excessive excitational neuronal transmitters, production of free radicals, inhibition of protein synthesis, abnormal expression of genes, and activation of immune responses. However, there have been no therapeutically effective agents developed to protect against damage to brain neuronal cells.

Ginseng (the root of \textit{Panax ginseng} C. A. Meyer, Araliaceae) is frequently used as a crude drug and is taken orally as a traditional medicine in Asian countries. The major components of ginseng are the ginsenosides Rb1, Rb2, and Re, which are glycosides with a dammarane skeleton.\(^{1,2}\) When ginseng is steamed at 98–100 °C, it changes into red ginseng, of which the main components are ginsenoside Rg3 and ginsenoside Rb1.\(^{3}\) In addition, Han \textit{et al.} reported that when ginsenosides Rb1 and Rb2 were incubated under mildly acidic condition, these ginsenosides were transformed into ginsenoside Rg3.\(^{4}\) We also reported that ginsenoside Rc was transformed to ginsenoside Rg3 with the addition of diluted hydrochloric acid, lactic acid, or acetic acid.\(^{5}\) These results suggest that the ginsenosides can easily be transformed under acidic conditions and that the composition of the acid-treated ginseng (AG) may be similar to that of red ginseng.

These ginsenosides have been reported to show various biological activities, including antiinflammatory activity\(^{6}\) and antitumor effects.\(^{7,8}\) To explain these pharmacologic actions, it is thought that ginseng saponins must be metabolized by human intestinal bacteria after being taken orally.\(^{9–12}\) Ginsenosides Rb1, Rb2, and Re are transformed to 20-\( \beta \)-D-glucopyranosyl-20(S)-protopanaxadiol (IH-901, compound K) by human intestinal bacteria. The IH-901 induces an antitumorigenic effect by blocking tumor invasion.\(^{13}\) Ginsenoside Rg3, which is the main component of red ginseng or AG, was metabolized to ginsenoside Rh2 by human intestinal bacteria.\(^{5}\) The transformed ginsenoside Rh2 showed more potent cytotoxic activity than ginsenoside Rg3. However, the inhibitory effects of ginseng and ginsenosides on ischemic brain injury have been not studied.

Therefore ginseng treated with or without mild acid was incubated with human intestinal microflora, and the inhibitory effect of the main metabolite ginsenoside Rh2 of AG on the ischemic brain injury induced by a transient focal ischemic rat model was investigated.

MATERIALS AND METHODS

Materials, Cells, and Fecal Microflora  The lipopolysaccharide (LPS), prostaglandin-E\(_2\) (PGE\(_2\)) assay kit, 2,3,4-triphenyltetrazolium chloride (TTC), hexadecyltrimethyl ammonium bromide and ortho-dianisidine were purchased from Sigma Chemical Co. (St. Louis, MO, U.S.A.). Isoflurane was purchased from Choongwae Pharmaceutical Co., Ltd. (Korea), and the general anaerobic medium (GAM) was from Nissui Pharmaceutical Co., Ltd. (Japan). All other chemicals were of analytical reagent grade. The ginsenosides Rb1 and Rb2 were isolated from ginseng according to the previously described method.\(^{14}\) The 20(S)-ginsenoside Rg3 and 20(S)-ginsenoside Rh2 were prepared by our previously reported method.\(^{5}\)

Murine macrophage RAW264.7 cells were purchased from the Korea Cell Collection Bank. Human intestinal microflora were prepared as follows: 5 g of fresh feces (from a healthy man in his 20 s) was suspended in 100 ml of GAM broth and centrifuged at 500×\( g \) for 10 min, and the supernatant precipitated by centrifugation at 5000×\( g \) for 30 min and was used as a human fecal specimen.

Preparation of Ginseng and AG  The dried ginseng (0.5 kg) was crushed, extracted with MeOH 41, concentrated, suspended in distilled water, and extracted with BuOH 1.51 and the BuOH extract was concentrated (GB fraction). AG was prepared as follows: ginseng (0.5 kg) was extracted with MeOH 41, and then the MeOH extract was concentrated, suspended in 0.51 of 0.1% HCl, heated at 60 °C for 5 h, neutralized with 0.1% NaOH, and extracted with BuOH 2.51, and the BuOH extract was concentrated (AG fraction). Each concentrate was used as a sample. The assay for the ginsenoside contents of each sample was as follows: The sample (0.5 g) was suspended in 10 ml of distilled water, extracted with BuOH 20 ml, and assayed using an HPLC system consisting of a column, Lichrosorb NH\(_2\) column (25×0.4 cm, 5 µm, Merck Co., Germany); the mobile phase was a 94.9:9:0.1 (v/v/v) mixture of acetonitrile–water–isopropanol, at a flow rate of 1 ml/min, with a UV detector set at 208 nm.

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Fermentation and Metabolite Assay of Ginseng and AG by Human Intestinal Microflora

The incubation mixture containing 0.1% of each ginseng extract and fresh human fecal suspension (1 g) in a final 100-ml volume of anaerobic dilution medium according to the procedure of Hattori et al.15 was incubated for 24 h at 37 °C. The reaction mixture was extracted with BuOH 0.5 l. The BuOH extract was evaporated and assayed using TLC.

TLC TLC was performed as follows: TLC plates, silica gel 60F254 (Merck Co., U.S.A.); and developing solvents, chloroform–methanol–water 65 : 35 : 10 (v/v) and n-butanol–ethyl acetate–H2O 15 : 4 : 4 (v/v). The plates were stained by spraying with methanol–sulfuric acid=95 : 5 (v/v), followed by heating. The stained spots in TLC were then analyzed using a TLC scanner (Shimadzu, model CS-9301PC, Japan).

Assay of PGE2 RAW 264.7 cells were seeded at 5×10⁴ cells/well in flat-bottomed 96-well plates. The ginsenosides (or indomethacin) and LPS (1 µg/ml) were added to the culture medium, and the cells incubated at 37 °C for 20 h. The medium was collected in a microfuge tube and centrifuged at 2800 x g for 20 min. The supernatant was placed in a new microfuge tube and the amount of PGE2 determined using a PGE2 Enzyme-Immuno-Assay kit (Cayman Chemical).

Animals Male Sprague–Dawley rats (270—280 g) were purchased from the Charles River Branch of Biogenomics Co., Ltd. (Seoul, Korea). The rats were housed 5 per cage, allowed free access to water and food, and maintained at a constant temperature (22 ± 2 °C) and humidity (55 ± 10%), with a 12-h light/dark cycle (lights on 07:30—19:30 h). All procedures relating to animals and their care conformed to the International Guidelines: Principles of Laboratory Animal Care (NIH publication no. 85-23, revised 1985) and the Guiding Principles for the Care and Use of Laboratory Animals of Kyung Hee University, Korea.

Transient Cerebral Ischemia and Morphometric Measurement of Infarct Volume Rats were anesthetized in a chamber containing a mixture of N2O and O2 (70 : 30) and Diluted Hydrochloric Acid and/or with Intestinal Microflora. The incubation mixture containing 100 ml of 0.1% of each ginseng extract and fresh human fecal suspension (1 g) in a final 100 ml of anaerobic dilution medium was incubated for 24 h at 37 °C, extracted with BuOH, and the content of ginsenosides in the BuOH extract was analyzed by TLC.

RESULTS AND DISCUSSION

Transformation of Ginseng by Mild Acid and/or Human Intestinal Microflora Ginseng was incubated with 0.1% HCl at 60 °C, and then the content of transformed protopanaxadiol saponins were measured (Table 1). The contents of ginsenoside Rg3 and Δ20-ginsenoside Rg3 in mild acid-treated ginsengs were increased, compared with those of untreated ginseng. The main transformant was ginsenoside Rg3. This was supported by the previous reports of Han et al.14 and Bae et al.15 that ginsenosides Rb1 and Rb2 were transformed to ginsenoside Rg3 under mildly acidic conditions. The ginsenoside Rg3 also is a main component of red ginseng as well as 0.1% HCl-treated ginseng. When AG was incubated with human intestinal microflora, the ginsenoside Rg3 was decreased and ginsenoside Rh2 increased. This is also supported by the previous report of Bae et al. that ginsenoside Rg3 was metabolized to ginsenoside Rh2 by human

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a) The content of each ginsenoside per total ginsenosides is indicated. Each ginseng (0.5 g) was suspended in 10 ml of distilled water, extracted with 20 ml of BuOH, and BuOH extract was assayed by HPLC system: column, Lichrosorb NH2 (25×0.4 cm, 5 µm, Merck Co.); elution solvent, mixtures of solvent A (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—40 min; UV detector wavelength, 205 nm. b) Not treated with human intestinal microflora. c) Treated with human intestinal microflora. The incubation mixture containing 100 ml of 0.1% of each ginseng extract and fresh human fecal suspension (1 g) in a final 100 ml of anaerobic dilution medium was incubated for 24 h at 37 °C, extracted with BuOH, and the content of ginsenosides in the BuOH extract was analyzed by TLC.
intestinal bacteria. In addition, the ginsenosides Rb1, Rb2, and Rc were metabolized to compound K (IH-901) by human intestinal bacteria. Based on these findings, the pharmacologic actions of ginseng may be different from those of AG or red ginseng.

**Inhibitory Effect of Ginseng and Its Metabolite Ginsenoside Rh2 on Brain Injury Induced by Transient Focal in the Ischemia-Reperfusion Rat Model**  
The inhibitory effect of ginseng in the ischemia-reperfusion rat model was investigated (Fig. 1A). The occlusion of the MCA resulted in extensive and reproducible hemispheric swelling and focal infarction throughout the cortical and subcortical structures. A large infarct (total infarct volume 393.3 ± 38.1 mm³) was induced after 120 min of ischemia/22-h reperfusion in the control animals. The ginseng extract-treated group did not show a reduction in the infarct area in any region. However, the AG-treated group showed reductions in the infarct area in all regions, especially in the 3rd and 4th sections (data not shown), and the total infarct volume in this group was significantly lower than in the vehicle-treated controls (p < 0.05). The AG-treated group also exhibited lower myeloperoxidase activity than that observed in the control group (p < 0.05) (data not shown). Therefore, to screen for the antiischemic activity, although Yuan et al. reported that the orally administered main component of AG, ginsenoside Rg3, is metabolized to ginsenoside Rh2 by the intestinal bacteria and that ginsenoside Rh2 can inhibit ischemic brain injury caused by inflammatory reactions. Finally, we suggest that AG could be a prodrug, of which the biotransformant ginsenoside Rh2 can improve ischemic brain injury.

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**REFERENCES**