20(S)-Ginsenoside Rg3 Enhances Glucose-Stimulated Insulin Secretion and Activates AMPK

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Although Panax ginseng has been widely used in oriental countries for pharmacological effects such as anti-diabetic, anti-inflammatory, adaptogenic, and anti-fatigue activities, the active ingredient is not yet fully identified. In our preliminary studies, protopanaxadiol ginsenosides showed the insulin secretion-stimulating activity. In HIT-T15 cells, Rg3 enhanced the insulin secretion in a concentration dependent manner. This effect, however, was almost completely abolished in the presence of diazoxide (K⁺ channel opener) or nifedipine (Ca²⁺ channel blocker). Oral glucose tolerance test (OGTT) was also performed using ICR mice and Rg3 suppressed the blood glucose levels from rising by enhancing an insulin secretion at 30 min after administration. From these studies, we may conclude that Rg3 lowered the plasma glucose level by stimulating an insulin secretion and this action was presumably associated with ATP sensitive K⁺ channel. Next, to explore the hypothesis that ginsenoside Rg3 epimers may exhibit differential effects, glucose-stimulated insulin secretion activity and phosphorylation of AMP-activated protein kinase (AMPK) were compared between 20(S)- and 20(R)-ginsenoside Rg3. 5 μM of 20(S)-Rg3 enhanced the glucose-stimulated insulin secretion by 58% compared to the control, but 20(R)-Rg3 did not show any effect. In C2C12 myotubes, 20(S)- and 20(R)-Rg3 both markedly phosphorylated AMPK and acetyl-CoA carboxylase (ACC), although 20(R)-Rg3 showed a little less effect. Taken together, our results suggest that ginsenoside Rg3 epimers showed differential activities, and 20(S)-Rg3 epimer exhibited the higher pharmacological effects in insulin secretion and AMPK activation than 20(R)-Rg3. The novel characteristics of 20(S)-Rg3 may be a valuable candidate for anti-diabetic agent.

Key words 20(S)-ginsenoside Rg3; diabetes; ATP sensitive K⁺ channel; oral glucose tolerance test; AMP-activated protein kinase

Diabetes mellitus is a serious metabolic disease affecting major populations worldwide. The number of people with diabetes is anticipated to rise from current estimate of 150 to 220 million in 2010, and 300 million in 2025. Lifestyle in industrialized societies such as high caloric-diet and sedentary lifestyle gives the fundamental causes of this fast-spread ‘epidemic’. Diabetes mellitus is divided into two main forms. Type 1 diabetes mellitus (T1DM) is mainly due to an autoimmune-mediated destruction of pancreatic β-cell islets. On the other hand, type 2 diabetes mellitus (T2DM) is characterized by insufficient insulin secretion and insulin resistance. Epidemiological studies and clinical trials strongly support the notion that hyperglycemia is the principal cause of microvascular and macrovascular complications. Therefore, effective blood glucose control is the key to preventing or reversing diabetic complications and improving quality of life in diabetic patients. Although no cure is yet available for T2DM, oral hypoglycemic agents have been developed and are widely used. These therapies, however, are not perfect and characterized by insufficient efficacy, limited tolerability, or significant mechanism-based adverse effects. Therefore, novel treatment options are urgently needed that take advantage of physiological regulatory mechanisms and that result in weight loss or lack of weight gain.

Ginseng has been used as tonic and restorative remedies in traditional Chinese medicine for several thousand years. The pharmacological properties of ginseng are mainly attributed to ginsenosides, which are the active components found in the extracts of different species of ginseng. There have been plenty of studies demonstrating the anti-diabetic activity of ginsenosides, however, the active component with anti-diabetic activity is yet to be identified. In our preliminary study, protopanaxadiol ginsenosides potentiated an insulin secretion stimulated by a low concentration of glucose. Ginsenoside Rg3, 3β,12β,20-dihydroxydammar-24-ene 3-O-[β-D-glucopyranosyl (1→2)-β-D-glucopyranosyl] (Fig. 1), showed the most potent insulin secretion-stimulating activity. Ginsenoside Rg3 has been shown to inhibit tumor metastasis in mice as well as the invasion and metastasis of several tumors of rats and human in vitro. However, anti-diabetic effect of Rg3 has not been studied elsewhere as of this writing. Therefore, it would be interesting to examine whether and how Rg3 has anti-diabetic activity. To investigate anti-diabetic activity of Rg3, firstly we examined the insulin secretion-stimulating activity of Rg3 using HIT-T15 cells, followed by the oral glucose tolerance test using ICR mice. Next, we explored whether Rg3 activates an AMP-activated protein kinase (AMPK), a master switch regulating glucose and lipid metabolism, in C2C12 myotubes.

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MATERIALS AND METHODS

Drug and chemicals  Rg3 was obtained from the Central Research Center, ILHWA Pharmaceutical Co. (Guri, Korea). Rg3 was dissolved in 0.1% dimethyl sulfoxide (DMSO). Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), Roswell Park Memorial Institute (RPMI), penicillin, streptomycin, and heat-inactivated fetal bovine serum were purchased from WelGENE Inc. (Seoul, Korea). Diazoxide, digitonin, EDTA, glipizide, glucose, HEPES, nifedipine, phenylmethanesulfonyl fluoride (PMSF), sucrose and Tris–HCl were purchased from Sigma (St. Louis, U.S.A.). The phosphospecific-AMPK Thr172, phosphospecific-acetyl-CoA carboxylase (ACC) and extracellular regulated kinase (ERK) antibodies were obtained from Cell Signaling Technology (Danvers, U.S.A.). Rat and mouse insulin enzyme immunoassay ELISA kits were bought from Shibayagi (Gunma, Japan). Other reagents were of the highest purity commercially available.

Animal Our study was reviewed and approved by the Animal Care and Use Committee of Kyung Hee University. Five-week-old male ICR mice were purchased from ORIENT BIO (Sungnam-si, Korea), and they were acclimatized as previously described. 12) The animals were housed in individual cages for 2 weeks before being randomly assigned into the experimental groups. The animals were fed with standard rodent chow (LabDiet, Richmond, U.S.A.) and water ad libitum.

HIT-T15 Cells Culture Hamster pancreatic β-cell line, HIT-T15, was obtained from ATCC Global Bioresource Center. HIT-T15 cells (between passages 65—70) were cultured in RPMI 1640 media containing 11.1 mM glucose with 10% fetal bovine serum (FBS), 100 U/ml penicillin and 100 μg/ml streptomycin. Media was changed every 2 d and cells were subcultured every 5—6 d.

Insulin Secretion HIT-T15 cells were seeded into 24-well plate at a density of 2×10^5 cells per well and grown for 24 h. The cells were washed twice and preincubated for 30 min in Krebs–Ringers Bicarbonate (KRB) buffer [115 mM NaCl, 4.7 mM KCl, 2.56 mM CaCl_2, 1.2 mM KH_2PO_4, 1.2 mM MgSO_4, 20 mM NaHCO_3, 16 mM HEPES and 0.3% bovine serum albumin, pH 7.4]. Cells were then treated with KRB buffer containing 5 mM glucose with or without Rg3, and incubated for 1 h at 37°C. After incubation, aliquots of the media were stored at −20°C until insulin measurement. To explore how Rg3 augments the glucose-stimulated insulin secretion, HIT-T15 cells were incubated for 1 h in KRB buffer containing either 0.5 mM diazoxide (K^+ channel opener) or 10 μM nifedipine (L-type of Ca^{2+} channel blocker) in the absence or presence of Rg3 (8 μM), and insulin concentration was measured.

Oral Glucose Tolerance Test (OGTT) The ICR mice were fasted for 12 h prior to the experiment, and Rg3 (12.5, 25 mg/kg body weight) was administered orally 30 min prior to glucose challenge. Glucose (1.5 g/kg) was orally administered at 0 min, and the blood was withdrawn from the orbital venous plexus at 0, 30, 60 and 120 min after glucose administration. Plasma glucose and insulin levels were determined by the glucose oxidase method and mouse insulin ELISA kit, respectively.

Protein Extract and Western Blot The skeletal muscle cell line C2C12 myoblasts were maintained in DMEM supplemented with 10% heat-inactivated FBS at 37°C with 95% air and 5% CO_2. Differentiation into myotubes was performed as previously described. 12) To induce differentiation, media was replaced with DMEM containing 1% FBS when they are confluent. Experiments were performed in differentiated C2C12 myotubes after 7 d in 1% FBS/DMEM.

C2C12 myotubes were incubated for 1 h with 10, 50, 100 μM of Rg3. Cells were lysed with extraction buffer (50 mM Tris–HCl, pH 7.5, 1 mM EDTA, 0.25% sucrose, 0.4 mg/ml digitonin, and 1.5 mM PMSF). The plate was rocked on ice for 3 min, and the buffer was collected for Western blot. The proteins in the cytosolic fraction were separated on a 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), transferred to a nitrocellulose membrane, and probed using horseradish-peroxidase conjugates. ERK protein levels were used as a control for equal protein loading.

Statistical Analysis Data were analyzed using a one way ANOVA, followed by Turkey’s post hoc test for multiple comparisons. A p value of <0.05 was considered significant.

RESULTS AND DISCUSSION

To explore whether Rg3 augments a glucose-stimulated insulin secretion, different concentrations of Rg3 were treated to HIT-T15 cells. In HIT-T15 cells, Rg3 at the concentration range between 2 and 8 μM augmented a glucose-stimulated insulin secretion in a concentration dependent manner with

![Fig. 2. Effect of Rg3 on Insulin Secretion](image)

Insulin concentration was measured in HIT-T15 cells treated with Rg3 at different doses (A), and in the presence of diazoxide (B) or nifedipine (C). Values were means±S.E. *p<0.05, **p<0.01, ***p<0.001 compared to control.
the maximal response occurring at 8 \( \mu \text{M} \) (Fig. 2A). 16 \( \mu \text{M} \) of Rg3 also stimulated the insulin secretion, but magnitude was smaller than that in 8 \( \mu \text{M} \) of Rg3. Next, to examine how Rg3 enhances a glucose-stimulated insulin secretion, diazoxide (K^+ channel opener) and nifedipine (L-type of Ca^{2+} channel blocker) were used. Diazoxide (0.5 \text{mM}) blocked a glucose-induced insulin secretion from 100\( \pm \)7.3 to 55.6\( \pm \)7.2 \(\mu\text{U/ml}\) in HIT-T15 cells (\(p<0.05\), Fig. 2B). In HIT-T15 cells supplemented with 5 \(\text{mM}\) glucose and 8 \(\mu\text{M}\) Rg3, diazoxide suppressed the insulin secretion to a level observed in 5 \(\text{mM}\) glucose with diazoxide (\(p<0.001\)). The addition of 10 \(\mu\text{M}\) nifedipine also reduced the insulin secretory effect of Rg3 from 111.1\( \pm \)15.6 to 49.5\( \pm \)5.9 \(\mu\text{U/ml}\) (\(p<0.05\), Fig. 2C), to an extent comparable to 5 \(\text{mM}\) glucose and nifedipine. However, we cannot rule out the possible engagement of alternative cAMP-dependent pathway in insulin secretion stimulating activity of Rg3.

Oral glucose tolerance test (OGTT) was performed to determine the effect of a single oral dose of Rg3 on glucose tolerance and insulin secretion using the ICR mice (Fig. 3). Glucose challenge dramatically increased the blood glucose levels in control group mice, whereas Rg3-treated groups (12.5, 25 \text{mg/kg} dose) showed 6% and 9% (\(p<0.05\)) reduction, respectively, compared to that of control group (Fig. 3B). Plasma insulin level at 30 min after glucose load in control group was 21.3\( \pm \)0.9 \(\mu\text{U/ml}\), whereas insulin levels in Rg3L and Rg3H-treated groups were 24.2\( \pm \)1.0 (\(p<0.05\)) and 28.8\( \pm \)2.0 \(\mu\text{U/ml}\) (\(p<0.01\)), respectively, indicating that Rg3 lowered the blood glucose levels by enhancing insulin secretion (Fig. 3C). Based upon these results, we may conclude that Rg3 lowered the plasma glucose level by stimulating an insulin secretion and this action was presumably associated with ATP sensitive K^+ channel.

Next, to explore differential effects of ginsenoside Rg3 epimers, glucose-stimulated insulin secretion and phosphorylation of AMPK were examined in HIT-T15 and C2C12 myotubes, respectively, in the presence of several concentrations of 20(S)- and 20(R)-Rg3. Values were means\(\pm\)S.E. \(**p<0.01\) compared to control.

AMPK is considered as a master switch regulating glucose and lipid metabolism, and an enzyme that works as a fuel gauge, being activated in condition of high energy phosphate depletion.\(^{13,14}\) In skeletal muscle, AMPK activation results in enhanced fatty acid oxidation and glucose uptake into the cells via stimulating translocation of glucose transporter.\(^{15}\) Therefore, phosphorylations of AMPK and ACC were examined in Rg3 epimer-treated C2C12 myotubes, skeletal muscle cell line. 20(S)-Rg3 markedly promoted the phosphorylations of AMPK and ACC, and 20(R)-Rg3 showed a less effect (Fig. 4B). In skeletal muscle, fatty acid oxidation is regulated by AMPK-ACC-malonyl-CoA axis. ACC produces malonyl-CoA, which inhibits carnitine palmitoyltransferase I, the rate-limiting step in mitochondrial fatty acid oxidation. Thus, the activation of AMPK by ginsenoside Rg3 inhibits...
ACC, decreases malonyl-CoA levels, and leads to stimulation of fatty acid oxidation. There are two major mechanisms enhancing glucose transport in skeletal muscle. One is insulin signaling pathway via PI-3 kinase/Akt. The other is AMPK signaling pathway, which is activated by such as stimuli as exercise, hypoxia, and hyperosmolarity. Therefore, stimulation of glucose transporter translocation is expected by Rg3 treatment, and we are currently investigating this possibility.

In summary, our results suggest that epimerization of carbon-20 of ginsenoside Rg3 revealed differential activities, and 20(S)-Rg3 epimer exhibited the higher pharmacological effects in insulin secretion and AMPK activation than 20(R)-Rg3. The novel characteristics of 20(S)-Rg3 may be a valuable candidate for anti-diabetic agent.

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