Pectinase-Processed Ginseng Radix (GINST) Ameliorates Hyperglycemia and Hyperlipidemia in High Fat Diet-Fed ICR Mice

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Abstract
To develop a ginseng product possessing an efficacy for diabetes, ginseng radix ethanol extract was treated with pectinase and obtained the GINST. In the present study, we evaluate the beneficial effect of GINST on high fat diet (HFD)-induced hyperglycemia and hyperlipidemia and action mechanism(s) in ICR mice. The mice were randomly divided into five groups: regular diet group (RD), high fat diet group (HFD), HFD plus GINST at 75 mg/kg (GINST75), 150 mg/kg (GINST150), and 300 mg/kg (GINST300). Oral glucose tolerance test reveals that GINST improves the glucose tolerance after glucose challenge. Fasting plasma glucose and insulin levels were decreased by 4.3% and 4.2% in GINST75, 10.9% and 20.0% in GINST150, and 19.6% and 20.9% in GINST300 compared to those in HFD control group. Insulin resistance indices were also markedly decreased by 8.2% in GINST75, 28.7% in GINST150, and 36.4% in GINST300, compared to the HFD control group. Plasma triglyceride, total cholesterol and non-esterified fatty acid levels in the GINST300 group were decreased by 13.5%, 22.7% and 24.1%, respectively, compared to those in HFD control group. Enlarged adipocytes of HFD control group were markedly decreased in GINST-treated groups, and shrunken islets of HFD control mice were brought back to near normal shape in GINST300 group. Furthermore, GINST enhanced phosphorylation of AMP-activated protein kinase (AMPK) and glucose transporter 4 (GLUT4). In summary, GINST prevents HFD-induced hyperglycemia and hyperlipidemia through reducing insulin resistance via activating AMPK-GLUT4 pathways, and could be a potential therapeutic agent for type 2 diabetes.

Key Words: Pectinase-processed ginseng radix, High fat diet, Insulin resistance, AMP-activated protein kinase, Glucose transporter 4

INTRODUCTION
Diabetes mellitus (DM) is the most common endocrine disease (Kim et al., 2007; Tuttle et al., 2007). Worldwide, more than 194 million people are affected and this number is expected to grow further to approximately 333 million by 2025 (Liao et al., 2010; Zhu et al., 2010). High caloric diets and sedentary lifestyles in industrialized societies are fundamental causes of this fast-spread “epidemic”. Obesity is a well-recognized risk factor for type 2 diabetes when combined with other known risk factors (Niswender, 2010). It has been an important therapeutic goal to reduce the risk of type 2 diabetes through weight management. Numerous epidemiological studies showed that hyperglycemia and hyperlipidemia are the principal cause of cardiovascular diseases (Cusi, 2010; Niswender, 2010; Yamamoto et al., 2010). Therefore, effective blood glucose and lipid control are the key to preventing or reversing diabetes complications and improving quality of life in diabetic patients (Warren, 2004). Modern drugs, including insulin and other hypoglycemic agents such as biguanides, sulfonylureas etc. control the blood glucose level only when they are regularly administered, but these treatments are tedious and have several disadvantages (Kobayashi et al., 2000; Stades et al., 2004; Chiang et al., 2007).

Ginseng is a well-known medicinal plant widely used in oriental societies as one of the most valuable medicines. The ginseng root has been used as a health product or natural remedy for a long time. To develop a ginseng product with an efficacy for hyperglycemia and hyperlipidemia, ginseng radix was processed with pectinase enzyme to give us a ‘GINST’. Here, we investigate the anti-diabetic and anti-hyperlipidemic activities of GINST using high fat diet-fed ICR mice.
MATERIALS AND METHODS

Chemicals
Pectinase was purchased from the DSM food specialties (ZAE La Baume, Servian, France). Antibodies against AMP-activated protein kinase (AMPK), phospho-AMPK, acetyl-CoA carboxylase (ACC), phospho-ACC, glucose transporter 4 (GLUT4) were from Cell Signaling Technology (Beverly, MA, USA), and anti-actin, anti-insulin and anti-goat IgG-HRP were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Protein extraction and western blot detection kits were from Intron Biotechnology Inc (Beverly, MA, USA). Bio-Rad protein assay kit was from Bio-Rad Laboratories (Hercules, CA, USA). Polyvinylidine difluoride membrane was from Millipore (Bedford, MA, USA). All other chemicals were of analytical grade.

Preparation of GINST
GINST was obtained from ILHWA Pharmaceutical Co. LTD. (Guri, Korea). Briefly, the ginseng radix (containing with 30-40% moisture) was extracted with 40-50% ethanol and concentrated with a speed-vac, then incubated with an enzyme solution containing pectinase at 55°C for 24 h.

Analysis of ginsenosides in GINST
An acuity liquid chromatograph (Waters, Milford, MA, USA) equipped with gradient pump, autosampler, and diode array detection was used. An Acquity UPLC BEH C18 reversed-phase column (100×1.0 mm, i.d., 1.7 μm) was used. The mobile phase consisted of water (solvent A) and acetonitrile (solvent B). The gradient elution was used as follow: 0-3 min, 5% B; 10 min, 15% B; 12 min, 30% B; 15 min, 35% B; 20 min 60% B. The column temperature was kept constant at 35°C, and the flow rate was 0.5 ml/min.

Animals
Five-week-old ICR mice were purchased from ORIENT BIO (Seoul, Korea). All animals were acclimatized to the laboratory environment for 1 week before the experiment. Mice were allowed to freely access to drinking water and food under constant room temperature (22 ± 2°C) and humility (50 ± 10%) conditions with an automatic 12 h light and dark cycle and experimental protocol was approved by the Institutional Animal Ethics Committee of the Kyung Hee University. Mice were randomly divided into five groups as following; group fed a regular diet (RD), group fed a high fat diet (HFD, Research diet, New Brunswick, NJ, USA), treatment groups fed a high fat diet plus GINST at 75 mg/kg (GINST75), 150 mg/kg (GINST150), and 300 mg/kg (GINST300). GINST was orally administered once a day for five weeks and body weight was measured once a week.

Oral glucose tolerance test (OGTT)
At the end of the experiment, the ICR mice were fasted for 12 h prior to the experiment. Glucose (1.5 mg/kg) was orally administered at 0 min, and the blood was withdrawn from the orbital venous plexus at 0, 30, 60 and 90 min after glucose administration. Plasma glucose was determined by the glucose oxidase method.

Determination of serum parameters
At the end of treatment, the mice were fasted for 12 h and anaesthetized by diethyl ether and blood samples were collected by cardiac puncture. Blood samples were centrifuged at 3,000 g for 15 min at 4°C, and plasma glucose, triglyceride (TG), total cholesterol (TC) and non-esterified fatty acid (NEFA) levels were determined using commercial kits (Stanbio Laboratory, Boerne, TX, USA) and automatic analyzer (SMARTLAB, Mannheim, Germany). The plasma insulin concentrations were determined using a mouse insulin enzyme immunoassay kit (Shibayagi, Gunma, Japan).

Histological analysis
For hematoxylin-eosin (HE) staining, the pancreas and epididymal fat were removed and fixed in 10% neutral buffered formalin. The tissues were subsequently embedded in paraffin and sectioned with 5 μm thickness (Leica, Wetzlar, Germany) and stained with hematoxylin-eosin for microscopic assessment (Olympus, Tokyo, Japan). The number of islet cells was measured as previously described (Liu et al., 2009) with some minor modifications and graded as 1, 1-40 cells per section; 2, 41-100 cells per section; 3, 101-201 cells per section; 4, 201-400 cells per section; 5, >400 cells per section. To examine the insulin contents in pancreas, immunohistochemistry technique was used. The sections were deparaffinized in xylene and rehydrated through a graded ethanol series. Antigen retrieval was performed by 0.1% trypsin. To block nonspecific binding of immunoglobulin, the sections were incubated with normal serum blocking solution for 30 min at room temperature. Goat anti-insulin IgG (1:75) was applied overnight at 4°C, and then tissue sections were incubated with donkey anti-goat IgG-HRP (1:200) for 30 min at room temperature. Positive control was visualized DAB peroxide substrate solution for 5-10 min, and tissues were counterstained with hematoxylin.

Western blot analysis
To determine protein expression of AMPK and GLUT4 in skeletal muscle, femoral muscle was removed. Total protein extracts were prepared using a protein extraction kit and insoluble protein was removed by centrifugation at 13,000 g for 20 min. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit. For Western blotting, 40 μg of protein was separated by 8% SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes. The membrane was further incubated with the indicated primary antibody, followed by secondary antibody conjugated with horseradish peroxidase. Protein bands were detected using an enhanced chemiluminescence Western blotting detection kit and then exposed to X-ray film.

Statistical analysis
Data were presented as mean±SEM. Statistical analysis was performed using one-way ANOVA followed by student-newman keuls test. p<0.05 was considered significant.

RESULTS

UPLC analysis
UPLC chromatograms of untreated and pectinase-treated ginseng radix extracts are shown in Fig. 1. The saponin peaks in untreated ginseng radix (peak 3, 4, 5 and 6 represent ginsenoside Rb1, Rc, Rb2 and Rd, respectively) were decreased during the enzyme treatment. After 24 h of pectinase treatment, these four ginsenosides were difficult to identify in the
chromatogram (Fig. 1A). On the other hand, IH-901 (peak 7 in Fig. 1B) was appeared during the enzyme process. IH-901 is an intestinal metabolite of protopanaxadiol type ginsenoside and might be one of active components for pharmacological effects of GINST. Several groups have reported anti-cancer, anti-inflammation, and anti-diabetic activities of IH-901 (Lee et al., 2000; Choi et al., 2007; Yoon et al., 2007). Having this chromatogram we attempt to explore whether GINST shows anti-hyperglycemic and anti-hyperlipidemic effects in high fat diet-induced ICR mice.

**Effect of GINST on OGTT**

One of the major characteristics of insulin resistance is the impaired glucose tolerance. To examine the effect of multiple oral administration of GINST on glucose tolerance, OGTT was carried out at the end of the experiment. As shown in Fig. 2, glucose challenge dramatically increased the blood glucose levels in the HFD group, whereas GINST-treated groups significantly prevented the blood glucose levels from rising during 90 min after glucose challenge (Fig. 2A). When the area under the curve (AUC) was compared between groups, GINST prevented the glucose absorption in a dose-dependent manner (Fig. 2B).

**Effects of GINST on body weight and metabolic parameters**

Body weight and metabolic parameters related to diabetes and hyperlipidemia are shown in Table 1. Weight gains in RD and HFD control groups during 5-week period were 4.1±0.8 g and 8.3 ± 1.0 g, respectively. However, GINST-treated groups lowered weight gain when compared to the HFD control group. Compared to the HFD control group, weight gain was lowered by 9.6% in GINST75, 21.7% in GINST150, and 48.2% in GINST300. Fasting plasma glucose levels in GINST-treated groups showed a significant decrease by 19.6% (p<0.05) in GINST300 group, compared to the HFD control group. Plasma insulin levels were also significant decreased by 4.2% in GINST75, 20.0% (p<0.01) in GINST150, and 20.9% (p<0.01) in GINST300 group, compared to the HFD control group. With decreased plasma glucose and insulin levels, the insulin resistance index (HOMA-IR) values (Matthews et al., 1985) for GINST-treated groups were markedly decreased by 8.2% in GINST75, 28.7% (p<0.01) in GINST150, and 36.4% (p<0.01) in GINST300 group compared to the HFD control group. The plasma levels of TG, TC and NEFA levels were also decreased by 13.5% (p<0.001), 22.7% (p<0.01) and 24.1% (p<0.001), respectively, in the GINST300 group compared to those in the HFD control group.

**Histological analysis**

High fat diet produces enlarged adipocytes, but GINST prevented them from formation of large adipocytes dose-dependently (Fig. 3A, B). The pancreas islets of the HFD control group mice were degenerated, whereas mice treated with GINST preserved islet architecture (Fig. 3A, B). Shrunken of
islet architecture induced by high fat diet was protected by GINST treatment, and this result was confirmed by insulin immunostaining (counterstained with hematoxylin) (Fig. 3C). Insulin contents in GINST-treated groups were dose-dependently restored when compared to the HFD control group.

**DISCUSSION**

DM is a metabolic disorder characterized by chronic hyperglycemia. The management of diabetes without any side effect is still a challenge to the medical practice. Clinical evidence has suggested that appropriate use of traditional Chinese medicines with modern Western medicinal or mainstream antidiabetic drugs can prevent or ameliorate the development of diabetic complications. Many diabetic patients choose alternative therapeutic approaches such as herbal or traditional Chinese medicine along with the mainstream antidiabetic drugs because of their effectiveness and fewer side effects.

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. IH-901, an intestinal metabolite of panaxadiol ginsenosides, is considered as

**Effects of GINST on AMPK activation**

Numerous studies demonstrated that skeletal muscle AMPK is implicated in a variety of antidiabetic properties of exercise, including GLUT4 expression, glycogen regulation, fatty acid oxidation, and enhanced insulin sensitivity (Furugen et al., 2011; Zhang et al., 2011). Thus, we examined whether GINST activates AMPK through phosphorylation in the femoral muscle. As shown in Fig. 4, GINST stimulates the phosphorylation of AMPK in a dose-dependent manner. ACC and GLUT4 (immediate targets of AMPK) protein expressions were also enhanced in GINST-treated groups.

**Table 1. Effects of GINST on body weight gain and metabolic parameters**

|                      | RD  | HFD | GINST (mg/kg) | 75  | 150 | 300 |
|----------------------|-----|-----|---------------|-----|-----|-----|
| Body weight (g)      |     |     |               |     |     |     |
| Initial              | 32.7 ± 1.6 | 32.7 ± 1.1 | 32.7 ± 0.7 | 32.8 ± 0.8 | 32.7 ± 2.4 |
| Final                | 36.8 ± 1.5 | 41.1 ± 0.8** | 40.2 ± 1.0 | 39.2 ± 0.9 | 36.7 ± 2.5*** |
| Weight gain (g)      | 4.1 ± 0.8  | 8.3 ± 1.0**  | 7.5 ± 0.9  | 6.5 ± 0.4** | 4.0 ± 0.7*** |
| Epididymal fat (g)   | 1.0 ± 0.1  | 2.4 ± 0.1**  | 1.9 ± 0.3  | 1.8 ± 0.1*  | 1.5 ± 0.1**  |
| Energy intake (Kcal/day) | 13.1 ± 0.2 | 16.1 ± 0.9** | 15.7 ± 0.7 | 14.0 ± 0.6* | 13.1 ± 0.4*** |
| Glucose (mM)         | 6.5 ± 0.4  | 9.2 ± 1.0**  | 8.8 ± 0.4  | 8.2 ± 0.4   | 7.4 ± 0.2*   |
| Insulin (μU/ml)      | 31.8 ± 1.2 | 47.9 ± 3.5** | 45.9 ± 5.1 | 38.3 ± 5.4** | 37.9 ± 4.7** |
| HOMA-IR              | 9.1 ± 0.5  | 19.5 ± 1.9** | 17.9 ± 0.5 | 13.9 ± 1.0** | 12.4 ± 0.9*** |
| Plasma lipids        |       |      |               |     |     |     |
| TG (mg/dl)           | 93 ± 11   | 114 ± 21**  | 116 ± 14   | 104 ± 17*  | 99 ± 11***   |
| TC (mg/dl)           | 136 ± 13  | 206 ± 25*** | 187 ± 10   | 175 ± 18*  | 159 ± 16**   |
| NEFA (μEq/L)         | 2,253 ± 9 | 2,961 ± 350** | 2,526 ± 274* | 2,519 ± 244* | 2,248 ± 53*** |

Data are mean ± standard error (n=5). Homeostasis model assessment (HOMA)-insulin resistance (IR) was used to calculate an index of insulin resistance as insulin (μU/ml)×glucose (mM)/22.5. *p<0.05, **p<0.01, and ***p<0.001 compared to high fat diet control (HFD) group. **p<0.01, and ***p<0.001 compared to regular diet control (RD) group.

**Effects of GINST on AMPK activation**

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the main active constituent in GINST (Fig. 1). Recently, IH-901 has received increasing attention because various pharmacological actions including anti-cancer, anti-inflammation, and anti-diabetes were shown to be mediated by this compound (Lee et al., 2000; Choi et al., 2007; Yoon et al., 2007). Previously, our group reported that IH-901 significantly decreased the fasting blood glucose levels in C57BL/6KSJ db/db mice through insulin secretion and improved insulin resistance (Han et al., 2007; Yuan et al., 2011). In addition, IH-901 was shown to activate AMPK and affect lipid metabolism in insulin-resistant human HepG2 cells (Kim et al., 2009). Therefore, we expect that GINST having IH-901 as an active constituent shows anti-diabetic activities in HFD fed ICR mice.

Impaired insulin action on whole-body glucose uptake is a hallmark feature of type 2 DM. Defects in insulin signal transduction through the insulin-receptor substrate-1/phosphatidylinositol 3-kinase pathway are associated with reduced insulin-stimulated GLUT4 translocation and glucose transport activity in type 2 diabetic skeletal muscle (Ryder et al., 2001). Therefore, elucidation of the signaling pathways governing contraction-induced increases in skeletal muscle glucose uptake may provide new pharmacological targets for the treatment of individuals with type 2 diabetes. In this study, GLUT4 protein expression was dose-dependently enhanced in GINST-treated groups in the skeletal muscle. AMPK is emerging as a signaling intermediary that controls the use of glucose and fatty acids in skeletal muscle (Egawa et al., 2011). Moreover, in skeletal muscle cells, AMPK may be activated by contraction or 5-aminoimidazole-4-carboxamide-1-b-D-ribofuranoside (AICAR), leading to increase in glucose uptake (Musi et al., 2010). The role of adipose tissue and lipotoxicity in the pathogenesis of type 2 diabetes.

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Fig. 4. Effects of GINST on phosphorylated AMPK and GLUT4 protein expression in femoral muscle. *p<0.01 vs. HFO.
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