Anti-diabetic Effect and Mechanism of Korean Red Ginseng in C57BL/KsJ db/db Mice

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(Received July 3, 2008; Accepted September 1, 2008)

Abstract: The present study was designed to investigate the anti-diabetic effect and mechanism of Korean red ginseng in C57BL/KsJ db/db mice. The db/db mice were divided into three groups: diabetic control group (DC), Korean red ginseng group (KRG, 100 mg/kg) and metformin group (MET, 300 mg/kg), and treated with drugs once per day for 10 weeks. Compared to the DC group, fasting blood glucose levels were decreased by 19.8% in KRG-, 67.7% in MET-treated group. With decreased plasma glucose and insulin levels, the insulin resistance index of the KRG-treated group was reduced by 27.6% compared to the DC group. The HbA1c levels in KRG and MET-treated groups were also decreased by 11.0% and 18.9% compared to that of DC group, respectively. Plasma triglyceride and non-esterified fatty acid levels were decreased by 18.8% and 16.8%, respectively, and plasma adiponectin and leptin levels were increased by 20.6% and 12.1%, respectively, in the KRG-treated group compared to those in DC group. Histological analyses of the liver and fat tissue of mice treated with KRG revealed significantly decreased number of lipid droplets and decreased size of adipocytes compared to the DC group. From the pancreatic islet double-immunofluorescence staining, we observed KRG has increased insulin contents, but decreased glucagon production. To elucidate action mechanism of KRG, effects on AMP-activated protein kinase (AMPK) and its downstream target proteins responsible for fatty acid oxidation and gluconeogenesis were explored in the liver. KRG activated AMPK and acetyl-coA carboxylase (ACC) phosphorylations, resulting in stimulation of fatty acid oxidation. KRG also caused to down regulation of SREBP1a and its target gene expressions such as FAS, SCD1 and GPAT. In summary, our results suggest that KRG exerted the anti-diabetic effect through AMPK activation in the liver of db/db mice.

Key words: Korean red ginseng, C57BL/KsJ db/db mice, diabetes, AMPK, fatty acid oxidation.

INTRODUCTION

Diabetes mellitus is the most common endocrine disorder characterized by hyperglycemia and long-term complications affecting the eye, kidney, nerve and blood vessel.1, 2) This disorder affects nearly 10% of the world’s population.3) The management of diabetes is considered a global problem and a cure has yet to be discovered. Modern drugs, including insulin and other hyperglycemia agents such as biguanides, sulphonylureas etc. control the blood glucose level only when they are regularly administered, but these treatments are tedious and have several disadvantages.4-6)

Plants have long been used as therapeutic purposes, and many of the currently available drugs are directly or indirectly derived from plants. Following the recommendations of the World Health Organization Expert Committee on Diabetes Mellitus, it is important to investigate hypoglycemic agents of plant origin used in traditional medicine.7)

Ginseng is a well-known medical plant used in traditional oriental medicine. The root of ginseng has been used as health product or natural remedy for a long time.8) Korean red ginseng (KRG), steamed root of Panax ginseng C.A. Meyer, has been known to have potent biological activities such as radical scavenging, vasodilating, anti-tumor and anti-diabetic activities.9-13) However, the mechanism of the beneficial effects of KRG on diabetes is yet to be elucidated. Therefore, in the present study, we investigated the anti-diabetic effect and action mechanism of KRG extract in C57BL/KsJ db/db mice.
MATERIALS AND METHODS

Materials KRG water extract was donated from KT&G (Seoul, Korea). The extracts 10 g were diluted in the 2 L water, freeze dried and kept at deep freezer until use.

Animals Five-week old C57BL/KsJ db/db mice were purchased from ORIENT BIO (Sungnam, Korea). All animals were acclimatized to the laboratory environment for 2 weeks before the experiment. Mice were allowed to freely access to drinking water and food under constant room temperature (22 ± 2°C) and humidity (50 ± 10%) conditions with an automatic 12 hr light and 12 hr dark cycle and cared for and treated in accordance with guidelines of the Committee on Care and Use of Laboratory Animal Resources, National Research Council, USA. Mice were randomly divided into three groups: diabetic control group (DC), KRG group (100 mg/kg), metformin group (MET, 300 mg/kg), and treated with drugs by oral administration once per day for 10 weeks. For oral administration, the drugs were diluted in the distilled water. During the experiment, body weight and blood glucose levels were measured once every week.

Assay of plasma parameters After 10 weeks treatment, the blood sample were collected and then centrifuged at 3,000g for 15 min at 4°C, and serum glucose, insulin, hemoglobin A1c (HbA1c), triglyceride (TG), non-esterified fatty acid (NEFA), adiponectin and leptin level were measured. Serum glucose concentration was determined using the glucose oxidase method (Asan Pharmaceutical Co., Korea). Serum insulin concentrations were determined using a mouse insulin enzyme immunoassay kit (Gunma, Japan). HbA1c was measured using a hemoglobin A1c kit (Biosystems S.A., Barcelona, Spain). Serum NEFA and adiponectin levels were determined using enzymatic colorimetric method (Eiken, Tokyo, Japan) and a mouse adiponectin ELISA kit (Adipogen, Korea), respectively. Leptin level was measured using a mouse leptin enzyme immunoassay kit (Linco Research, USA).

Histological analysis The pancreas, epidydimal fat tissue and liver 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, Japan). To examine the insulin and glucagon contents in pancreas on the same sections, a double immunofluorescence 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 Ig G (1:75, Santa Cruz Biotechnology, Santa Cruz, USA) were applied overnight at 4°C, followed the tissue sections were incubated with donkey anti-goat IgG-FITC (1:200, Santa Cruz Biotechnology, Santa Cruz, USA) for 30 min at room temperature. Then rabbit anti-glucagon Ig G (1:75, Santa Cruz Biotechnology, Santa Cruz, USA) were applied overnight at 4°C, followed the tissue sections were incubated with goat anti- rabbit Ig G-TR (1:200, Santa Cruz Biotechnology, Santa Cruz, USA) for 30 min at room temperature. Double immunolabeling was detected with the aid of a fluorescence microscope (AX-70, Olympus, Tokyo, Japan).

Western blot analysis After sacrificed, liver was immediately removed and instantly soaked in liquid nitrogen and stored at -70°C. Protein extracts were prepared in protein extraction kit (Intron Biotechnology Inc., Seoul, Korea). Lysates (30 µg) were electroblotted onto a nitrocellulose membrane following separation on a 8% SDS-polyacrylamide gel electrophoresis. Blotted membranes were incubated for 1 hr with blocking solution (tris-buffered saline/Tween 20, TBST) containing 5% skin milk (w/v) at room temperature, followed by incubation overnight at 4°C with 1:2000 dilution of AMP-activated protein kinase (AMPK), phospho-AMP-activated protein kinase (p-AMPK), acetyl-coA carboxylase (ACC), phospho- acetyl-coA carboxylase(p-ACC) primary antibody (Cellsignaling, USA). Membranes were washed four times with 0.1% TBST and incubated with 1:3000 dilution of horseradish peroxidase-conjugated goat anti-rabbit or donkey anti-rabbit IgG secondary antibody for 1 hr at room temperature. Membranes were washed four times in TBST and then developed by ECL (Amersham, Sweden).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR) Total mRNA was isolated from mice liver using an Easy-Blue kit (Intron Biotechnology Inc, Seoul, Korea) according to the manufacture’s instructions. From each sample, total RNA (10 µg) was reverse transcribed
into cDNA using the Moloney murine leukemia virus transcriptase and Oligo (dT) 15 primers (Promega, USA) as primers. The cDNA fragment was amplified by PCR using the following specific primers:

\[ \text{SREBP1a (Sterol regulatory element-binding proteins):} \]
\[ \text{sense 5'-GCG CTA CCG GTC TTC TAT CA-3', anti-} \]
\[ \text{sense 5'-TGC TGC CAA AAG ACA AGG G-3'; FAS (Fatty acid synthesis) sense 5'-GAT CCT GGA AGC AGA ACA C-3', anti-} \]
\[ \text{sense 5'-AGA CTG TGG AAC AGC GTG GT-3'; SCD1 (Stearoyl CoA desaturase) sense 5'-CGA GGG TTG GTT GTT GAT CTG T-3', anti-} \]
\[ \text{sense 5'-ATA GCA CTG TTG GCC CTG GA-3'; GPAT (Glycerol-3-phosphate acyltransferase) sense 5'-GGT AGT GGA TAC TCT GTC GTG GT-3', anti-sense 5'-CAT CAG CAA CAT TCG CF-3'; CD36 sense 5'-TCC TCT GAC ATT TGC AGG TCT ATC-3', anti-sense 5'- GTG AAT CCA GTT ATG GGT TCC AC-3'; PPAR-\alpha (Peroxisome proliferator activated receptor-\alpha) sense 5'-GCC CAG AGA TTT GAG GTC CT-3', anti-sense 5'-TTA GAG TTG TCC ACA GTC GGA GA-3'; PEPCK (Phosphoenolpyruvate carboxykinase) sense 5'-ATG CCT CCT CAG CTG CAT A-3', anti-sense 5'-TTA CAT CTG GCT CAT TCT CGT TT-3'.} \]

For SREBP1a, FAS, PPAR-\alpha, CPN, PEPCK, PCR was initiated a thermal cycle programmed at 95°C for 5min, 95°C for 30 sec, 57°C for 30 sec, 72°C for 30 sec, and amplified for 30 cycles. The RT-PCR products were electrophoresed on 1% agarose gels and visualized by 0.5ug/ml ethidium bromide staining and UV irradiation. CPN was amplified as a control gene.

**Statistical analysis** Data are expressed as mean values ± S.E. and comparisons of data have been done by unpaired Student’s t-test or ANOVA, as appropriate. Mean values were considered significantly different when \( p<0.05. \)

**RESULTS AND DISCUSSION**

**Effect of KRG on metabolic parameters** Table 1 shows the effects of KRG and MET on metabolic parameters in diabetic db/db mice treated for 10 weeks. Compared to DC, there were no significant differences in body weight between DC and KRG and MET group. KRG group showed increase in food intake and water intake, but MET group showed decrease in food intake and water intake. Plasma glucose levels were significant decreased by 19.6\% (\( p<0.05 \)) in KRG, 67.7\% (\( p<0.0001 \)) in MET compared to DC. Plasma insulin levels were also decreased by 10.0\% in KRG, 40.6\% (\( p<0.05 \)) in MET compared to DC. With decreased plasma glucose and insulin levels, the insulin resistance index (HOMA-IR)\(^{14} \) of the KRG group was reduced by 27.6\%, when compared to the DC group. HOMA-IR values for positive drug were markedly decreased by 80.8\% compared to diabetic control group.

Glycated hemoglobin (GHb) is a gold standard in biochemical analysis, and is essential to ensure the optimal care of diabetic patients.\(^{15} \) For hemoglobin, the rate of synthesis of

| Table 1. Metabolic parameters in Korean red ginseng treated db/db mice |
|-----------------|-----------------|-----------------|
| parameter       | DC              | KRG             | MET             |
| Body weight (g) | 56.3±1.04       | 56.1±1.25       | 56.1±1.25       |
| Food intake (g/mouse) | 373.01       | 378.38          | 349.26          |
| Water intake (ml/mouse) | 413.43          | 438.29          | 313.33          |
| Plasma glucose (mM) | 15.8±0.9        | 12.7±1.3*       | 5.1±0.4***      |
| Plasma insulin (\( \mu \)U/ml) | 240.6±57.1      | 216.6±18.2      | 143.0±37.0*     |
| HOMA-IR         | 168.9±46.3      | 122.3±30.0      | 32.4±13.0*      |
| HbA1c (%)       | 7.0±0.19        | 6.2±0.18*       | 5.6±0.18**      |
| Plasma adiponectin (\( \mu \)g/ml) | 31.5±1.6       | 38.0±1.3*       | 29.5±2.3        |
| Plasma leptin (ng/ml) | 54.5±1.3        | 61.1±1.1**      | 57.6±1.1        |
| TG (mg/ml)      | 94.9±9.4        | 77.0±1.6        | 65.4±4.9*       |
| NEFA (\( \mu \)eqiv/L) | 2179.3±78.0     | 1812.8±44.7*** | 1936.3±204.5    |

Data are mean ± standard error (n=4). Homeostasis model assessment was used to calculate an index of insulin resistance as insulin (\( \mu \)U/ml)xglucose (mM)/22.5. \( *p<0.05. \) \( **p<0.001 \) compared to diabetic control (DC) group.
GHb is principally a function of the concentration of glucose to which the erythrocytes are exposed. In our result, HbA1c levels in KRG- and MET- treated groups were decreased by 11.0% and 18.9%, when compared to DC group, and these data revealed a similar trend shown in plasma glucose levels.

Adiponectin is an adipokine secreted from adipose tissue. This adipokine has attracted much attention because of its multiple beneficial effects on a cluster of obesity-related metabolic and cardiovascular dysfunctions. Adiponectin exerts its pleiotropic beneficial effects through its direct actions on multiple target tissues. In the liver, only full-length adiponectin activates AMPK, thereby reducing molecules involved in gluconeogenesis and increasing phosphorylation of acyl-CoA carboxylase and fatty acid oxidation. Adiponectin activates peroxisome proliferator-activated receptor (PPAR), thereby stimulating fatty acid oxidation and decreasing tissue TG content in the liver. These alterations all increase insulin sensitivity in vivo. Therefore, elevating blood adiponectin and leptin (another adipokine improving insulin resistance) levels give us a direct evidence to increase the insulin sensitivity in vivo. In the KRG- treated group, adiponectin and leptin levels were both enhanced by 20.6% (p<0.05), and 12.1% (p<0.001), respectively, compared with DC group.

Plasma NEFA is primarily an important energy substrate for a number of organs. NEFA is also precursors for the formation of TG stores in adipose tissue, liver and muscle through esterification. Lipolysis in adipocytes is repressed by insulin, so it is normal in the fasted state when insulin levels are low. Insulin resistance, however, is also associated with lipolysis and NEFA release into the circulation. Elevated serum NEFAs inhibit insulin's ability to promote peripheral glucose uptake into muscle and fat to reduce hepatic glucose production. In the KRG-treated group, the TG and NEFA levels were lowered by 18.8% and 16.8%, respectively, compared with DC group. These results suggested that KRG caused a positive influence on glucose homeostasis.

**Histological analysis** Non alcoholic fatty liver disease, generally termed NAFLD, is an increasingly recognized health problem. Increased fat accumulation in the liver is observed in 20-30% of the population in the Western world. NAFLD is associated closely with metabolic disorders associated with insulin resistance, especially obesity and type 2 diabetes. But the cause of fatty liver is currently unknown. Histological analysis of the liver from the KRG-treated group mice revealed a significantly decreased number of lipid droplets compared with the DC group (Fig. 1). This result suggested that KRG not only controls blood glucose levels, but also has excellent effect on the NAFLD. Size of adipocytes in KRG-treated mice was also markedly decreased when compared to DC, as shown in Fig. 1.

**Detection of pancreas insulin and glucagon contents** In normal state when blood glucose level was increases, insulin synthesis is increased in β-cells, but glucagon production by the α-cells is suppressed. Conversely, when blood glucose level was decreased, β-cells secrete less insulin, glucagon secretion is increased, and hepatic glycogen stores are converted into glucose. From the pan-
creatic islet double immunofluorescence staining we observed KRG has increased insulin production and decreased glucagon production (Fig. 2).

**AMPK and ACC protein expression in the liver**
AMPK phosphorylation as measured by western blot in the liver. As shown in Fig. 3, KRG significantly phosphorylated AMPK and ACC compared to DC group. The phosphorylation of ACC decreases its activity and the concentration of malonyl CoA, the resulting product from the reaction, was decreased so that the activity of carnitine palmitoyltransferase (CPT1), which delivers fatty acid by using mitochondrial for fatty acid oxidation, was increased and thereby fatty acid oxidation was promoted.

Therefore, these results may elucidate the mechanism by which KRG promoted the fatty acid oxidation and inhibited the TG accumulation and enhanced the insulin sensitivity.

**Lipogenesis, lipolysis and gluconeogenesis gene expressions**
In general, activation of AMPK downregulates biosynthetic pathways such as fatty acid and cholesterol biosynthesis, yet switches on catabolic pathways that generate ATP, such as fatty acid oxidation, glucose uptake and glycolysis. With activation of AMPK, the expressions of target genes responsible for lipogenesis, lipolysis and gluconeogenesis were examined by RT-PCR. As shown in Fig. 4, KRG significantly inhibited the expressions of genes such as SREBP1α, FAS, SCD1 and GPAT, which are all associated with fat synthesis. In contrast, KRG significantly increased the expressions of lipolytic genes such as CD36 and PPAR-α. Interestingly, expression of phosphoenolpyruvate carboxykinase (PEPCK), a rate-limiting enzyme in gluconeogenesis pathway, was almost completely decreased compared to DC group.

From these results, we may conclude that KRG stimulated fatty acid β-oxidation through AMPK activation and ACC inactivation, and thereby inhibited the gene expressions of SREBP1α, FAS, SCD1, GPAT and inhibited the TG synthesis. Through AMPK activation and ACC inactivation, KRG also increased the gene expressions of lipolysis such as CD36, PPAR-α, and thereby stimulated fatty acid oxidation and decreasing TG content in the liver. Furthermore, through AMPK activation and ACC inactivation, KRG reduced hepatic glucose production.
through inhibiting PEPCK expression. As a result, KRG intercepted TG amassment and ameliorated the insulin resistant status, and then subsequently decreased the fasting glucose level.

These results suggest that KRG were similar with metformin in ameliorating insulin resistant status and decreasing the fasting glucose level in the obese type 2 diabetic patients, and did not have the lactic acidosis-like side effect with metformin at the meantime. Therefore, KRG has the prospect to be developed into an effective and safe blood sugar-lowering therapeutic agent.

ACKNOWLEDGEMENT

This work was supported by the grant from the Korean Society of Ginseng (2007).

REFERENCES


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