Fermented Ginseng Attenuates Hepatic Lipid Accumulation and Hyperglycemia through AMPK Activation

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Abstract - Fermented ginseng (FG) is an ethanol extract of ginseng radix processed with β-galactosidase. It was hypothesized that FG may exert anti-hyperlipidemic and anti-diabetic activities through modulating AMP-activated protein kinase (AMPK) in HepG2 human hepatoma cells. In this study, we showed that AMPK phosphorylation was stimulated by FG. These effects were abolished by pretreatment with an AMPK inhibitor, compound C. In addition, FG regulated the expression of genes associated with lipogenesis and lipolysis, thus causing suppression of hepatic triglyceride accumulation. In vivo study using db/db mice; FG reduced fasting plasma glucose, HbA1c, and insulin resistance index, when compared to diabetic control. FG also increased the phospho-AMPK and glucose transporter 4 (GLUT4) expressions in liver and skeletal muscle, respectively. In liver, expressions of lipogenic gene were decreased whereas expressions of lipolytic genes were induced, when compared to diabetic control. Taken together, we may suggest that FG ameliorates hyperglycemia and hyperlipidemia through activation of AMPK and could be developed as a health functional food or therapeutic agent for type 2 diabetic patients.

Keywords: fermented ginseng, AMP-activated protein kinase, db/db mouse, hyperglycemia, hyperlipidemia

Introduction

The basic defects in type 2 diabetes are insulin resistance of skeletal muscle, hyperinsulinemia in the early stage of the disease followed by insulin deficiency in the later stages, elevated hepatic glucose production, hyperglycemia, and dyslipidemia (1-3). These abnormalities in glucose and fat metabolism are often associated with obesity. Despite a considerable amount of effort and resources allocated to determining etiology, the basic cellular defect for the vast majority of diabetic patients remains undefined. The liver plays a major role in the regulation of glucose, lipid, and energy metabolism. Fatty liver and hepatic triglyceride accumulation are strongly associated with obesity, insulin resistance, type 2 diabetes, and subject to nutritional influences. Hepatic regulation of glucose and lipid homeostasis is influenced by a complex system of hormones, hormonally regulated signaling pathways, and transcription factors (4).

Recent data indicate that AMP-activated protein kinase (AMPK) has been implicated as a key regulator of energy dynamics, including gluconeogenesis, glucose uptake, and lipolysis (5-7). AMPK is a serine-threonine kinase activated following a rise in the intracellular AMP:ATP ratio. Numerous studies have described the effects of AMPK activation on liver metabolism (8,9). Two of the classical targets for the system are acetyl-CoA carboxylase (ACC) and 3-hydroxy-3-methylglutaryl-CoA reductase (HMGCR), catalyzing the key regulatory steps in fatty acid and sterol synthesis, respectively. The overall effects of AMPK activation in liver would be decreases in fatty acid, triglyceride, and sterol synthesis, and increases in fatty acid oxidation and ketogenesis.

Ginseng has been used as a tonic and restorative remedy in traditional Chinese medicine for several thousand years. The pharmacological effects of ginseng are mostly attributed to ginsenoside and many studies documented the anti-diabetic activity of ginsenoside (10-14). In a series of investigation to develop anti-diabetic agent with better efficacy from ginseng radix, fermented ginseng (FG) was developed. The objective of this study was to test the hypothesis that FG regulates hepatic lipid accumulation via AMPK signal pathways in HepG2 human hepatoma cells and we further examined its anti-diabetic activity and mechanisms using type 2 diabetic animal model, C57BL/6J db/db mice.

Materials and Methods

Preparation of fermented ginseng - Fermented ginseng was prepared as following: added ginseng radix ethanol extract and β-galactosidase (final concentration 1.5%) to distilled water, and incubated at 50°C for 72 hr, and adjusted the chilled solution with citric acid to pH 5.6, and finally incubated the solution with cellulase for 48 hr at 37°C until diol ginsenoside peaks were disappeared in ultra performance liquid chromatography (UPLC) chromatogram.

Analysis of ginsenosides in ginseng extracts - An Acquity liquid chromatograph (Waters Corporation, Milford, MA, USA) equipped with gradient pump, autosampler, and diode array detection was used. An acquity UPLC BEH C18 reverse-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 (15).

**Chemicals** Dulbecco’s modified Eagle’s medium (DMEM) and fetal bovine serum (FBS) were purchased from Gibco BL (Grand Island, NY, USA) and compound C was from Calbiochem (Darmstadt, Germany). Antibodies against AMPK, phospho-AMPK, phospho-LKB1, ACC, and phospho-ACC were from Cell Signaling Technology (Beverly, MA, USA), and anti-actin was from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Reverse transcriptase, Taq polymerase, and MTS solution were supplied by Promega (Madison, WI, USA). The polyvinylidene difluoride blotting membrane was from Millipore (Billerica, MA, USA) and ECL-reagent kit from Intron Biotechnology Inc. (Beverly, MA, USA). Infinity™ triacylglycerol reagent was from Asan Pharmaceutical Co. (Hwasung, Korea). Protein and RNA extraction kits were from Intron Biotechnology Inc. Other reagents and chemicals were of analytical grade.

**Cell culture and cell cytotoxicity assay** Human hepatoma HepG2 cell line was purchased from Korean Cell Line Bank (Seoul, Korea). HepG2 cells were cultured in DMEM containing 10% FBS, 100 unit/mL penicillin, 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO₂ at 37°C. For cell cytotoxicity assay, HepG2 cells were cultured in 96-well culture plate, and treated with indicated concentrations of FG for 24 hr. The cytotoxicity of FG was determined by CellTiter 96 AQ ueous One solution Cell Proliferation Assay kit (Promega, Madison, WI, USA).

**Determination of triglyceride content** Triglyceride content was determined in cell lysates using a colorimetric assay and expressed as µg of lipid/mg cellular protein as described (16). Triglyceride levels in cell lysates were measured according to the manufacturer’s instruction for Infinity™ triacylglycerol reagents.

**Blood sampling and plasma assay** Blood was withdrawn at the end of the experiment, using a heparinized capillary tube without anesthesia. The blood samples were placed on ice, centrifuged, and plasma stored at -70°C until assay. The plasma glucose concentration was determined using the glucose oxidase method. The plasma insulin concentration was measured according to the protocol described by the manufacturer of the mouse insulin enzyme linked immunosorbent assay (ELISA) kit (Shibayagi Co., Gunma, Japan). Plasma triglyceride was determined using commercially available kit (Asan Pharmaceutical Co., Seoul, Korea). Plasma non-esterified fatty acid (NEFA) concentrations were assayed by an enzymatic colorimetric method (Eiken Chemical Co., Tokyo, Japan). Glycated hemoglobin in whole blood was measured with a Hemoglobin A1c kit (BioSyt stems S.A., Barcelona, Spain). Plasma adiponectin level was measured according to the protocol described by the manufacturer of the mouse adiponectin ELISA kit (Adipogen, Seoul, Korea). Plasma leptin level was assayed by mouse leptin ELISA kit (Linco Research, MO, USA). Insulin resistance was determined by the homeostasis model assessment (HOMA) method by using the following equation: HOMA value for insulin resistance (HOMA-IR) = fasting insulin (µL/mL)× fasting glucose (mmol/L)/22.5 (17).

**Western blot analysis** Total protein extracts were prepared using a protein extraction kit and insoluble protein was removed by centrifugation at 12,000 × g for 20 min. Protein concentrations in cell lysates were measured using a Bio-Rad protein assay kit (Bio-Rad, Hercules, CA, USA). For Western blotting, 40-50 µg of protein was separated by 8% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to polyvinylidene difluoride membranes in a transfer buffer consisting of 20 mM Tris-HCl, 154 mM glycine, and 20% methanol. The membranes were blocked with 5% nonfat dry milk in Tris-buffered saline with 0.1% Tween 20 and incubated with specific antibodies and revealed with horseradish-peroxidase-conjugated secondary antibodies. The immunoreactive bands were visualized by an enhanced chemiluminescence and then were quantified by a densitometric analysis.

**RNA extraction and reverse transcriptase (RT) polymerase chain reaction (PCR)** Total RNA was isolated using an EASY-BLUE total RNA extraction kit (Intron Biotechnology Inc.) according to the manufacturer’s instruction. Single-strand cDNA synthesis was performed as described previously (18) using 5 µg of RNA, oligo (15)-dT primers and reverse transcriptase in total volume of 50 µL. PCR reactions were performed in a total volume of 20 µL comprising 2 µL of cDNA product, 0.2 mM of each dNTP, 20 pmol of each primer and 0.8 units of Taq polymerase. Oligonucleotide primer sequences used in PCR amplification were as follows; hGAPDH, forward 5'-TCCACCACCATGTTGCTGTA-3' and reverse 5'-ACCACGATCATGCACTAC-3'; hSREBP1c, forward 5'-TGGCCCGTCGATTTGAAGAC-3' and reverse 5'-ATGATACCCGAGGCGCATTCAG-3'; hEFS, forward 5'-CAAGAACTGCACGGAGGGTG-3' and reverse 5'-AGCTGCGAGATGGAGAAG-3'; hSCD1, forward 5'-TTGCCAGCTTCTAGCATTAAATTAC-3' and reverse 5'-
TCCTGGTAGCATTATTACGATTCCGGATTTTGCAGTT-3'; hCD36, forward 5'-GGGGCTATTAGGGAATCCATT-3' and reverse 5'-GGGCTATTAGGGAATCCATT-3'; hPPAR-α, forward 5'-TCCCCACTCGTCTTCCTTGATGTT-3' and reverse 5'-GCCCTGCCAAGGACAGGAGG-3'; mSREBP1a, forward 5'-TGCTGCCCAAAGACAGGAGG-3' and reverse 5'-GCCCTGCCAAGGACAGGAGG-3'; hSREBP1c, forward 5'-GGGCTATTAGGGAATCCATT-3' and reverse 5'-GGGCTATTAGGGAATCCATT-3'; mFAS, forward 5'-AGACTGTGGGAACACGGTGG-3' and reverse 5'-GAATACTGGGAGAACAC-3'; mSCD1, forward 5'-AAGCGAATCGTCTCCACGATTCCGGATTTTGCAGTT-3' and reverse 5'-AAGCGAATCGTCTCCACGATTCCGGATTTTGCAGTT-3'; mCPN, forward 5'-AGACTGTGGGAACACGGTGG-3' and reverse 5'-GGGCTATTAGGGAATCCATT-3'; mPPAR-α, forward 5'-AAGCGAATCGTCTCCACGATTCCGGATTTTGCAGTT-3' and reverse 5'-AAGCGAATCGTCTCCACGATTCCGGATTTTGCAGTT-3'; mCPN, forward 5'-AGACTGTGGGAACACGGTGG-3' and reverse 5'-GGGCTATTAGGGAATCCATT-3'.

PCR was performed at 95°C for 30 sec, followed by 50°C (PPAR-α), 52°C (hGAPDH), 56°C (hSCD1, hCD36, mSREBP1a, mFAS, mSCD1, mCD36, mPPAR-α, mCPN) or 64°C (hSREBP1c) for 30 sec, and 72°C for 1 min. The last cycle was followed by a final extension step at 72°C for 10 min. The RT-PCR products were electrophoresed in 0.8% agarose gels under 100 V and stained with 0.5 µg/mL ethidium bromide. Scanning densitometry was performed with I-MAX Gel Image analysis system (Core-Bio, Seoul, Korea).

**Results and Discussion**

Medicinal 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 WHO Expert Committee on Diabetes Mellitus, it is important to investigate hypoglycemic agents of plant origin used in traditional medicine. 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 (10-14). However, the active component responsible for anti-diabetic activity is yet to be identified. More concerning issue for utilizing a ginseng radix as a therapeutic agent is that ginseng radix showed a marginal anti-diabetic effect when compared to commercially available oral hypoglycemics. Therefore, we tried to develop a health functional food or therapeutic agent with better efficacy from ginseng radix.

**UPLC analysis** UPLC chromatograms of untreated and β-galactosidase treated ginseng radix extracts are shown in Fig. 1. The saponin peaks in untreated ginseng radix, ginsenoside Rb1, Rc, Rb2, and Rd were decreased during the enzyme treatment. After 72 hr of β-galactosidase treatment, these 4 ginsenosides were difficult to identify in
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Fig. 1B. The chromatogram showed compound K (peak 6) was appeared during the enzyme process. Compound K, an intestinal metabolite of panaxadiol ginsenosides, may be one of active components for pharmacological effects of FG. Recently, we reported that compound K significantly decreased the fasting blood glucose levels in C57BL/KsJ db/db mice through enhancing insulin secretion and improving insulin resistance (10). In addition, compound K was shown to stimulate AMPK phosphorylation in HepG2 and C2C12 cells (unpublished data). Having these preliminary results we attempt to explore whether FG stimulates AMPK activity in HepG2 cells and shows an anti-diabetic activity in C57BL/KsJ db/db mice.

**In vitro study/Effects of FG on cell cytotoxicity** To examine cellular toxicity of FG, various concentrations of FG (50-500 µg/mL) were treated on HepG2 cells for 24 hr. As shown in Fig. 2, FG did not show any cellular toxicity up to 500 µg/mL concentration and thus concentrations from 50 to 500 µg/mL of FG were employed in in vitro studies.

**In vitro study/Effects of FG on AMPK phosphorylation** To investigate whether phosphorylation of AMPK was induced by FG, HepG2 cells were treated with 500 µg/mL of FG for up to 24 hr (Fig. 3). After each time point, the cells were harvested and total cell lysates were extracted. The phosphorylation state of α subunit of AMPK (AMPKα) was noted by immunoblotting phospho-Thr-172 AMPK antibody. Compared to the basal level (0 time), FG markedly stimulated the phosphorylation of AMPK in a time dependent manner (Fig. 3A, 3B). No change in the expression of endogenous AMPKα was noted by immunoblotting AMPKα2 antibody. Being an immediate substrate of AMPK, we also examined whether ACC is phosphorylated by FG. As shown in Fig. 3A, ACC was significantly phosphorylated in a time dependent manner.

Fig. 2. Cellular toxicity of FG. Various concentrations of FG (50-500 µg/mL) were treated on HepG2 cells for 24 hr. Cell viability was measured by cell cytotoxicity assay.

Fig. 3. Effects of FG on AMPK and ACC phosphorylations in HepG2 cells. HepG2 cells were treated with FG 500 µg/mL for the indicated times (A) and the phosphorylation levels of AMPK and ACC were quantified and normalized to total AMPK protein (B). HepG2 cells were treated with different concentrations (100-500 µg/mL of FG for 24 hr (C). Effect of compound C on phosphorylations of AMPK and ACC (D). HepG2 cells were pretreated for 2 hr with compound C, and then treated with FG for additional 24 hr.
parallel with AMPK phosphorylation. Next, HepG2 cells were exposed to the indicated concentrations of FG for 24 hr, and AMPK and ACC were phosphorylated by FG in a dose dependent manner (Fig. 3C).

AMPK is a phylogenetically conserved serine/threonine protein kinase which has been proposed to act as a metabolic master switch in response to alterations in cellular energy charge. Once activated, AMPK leads to a concomitant inhibition of energy-consuming biosynthetic pathways and activation of ATP-producing catabolic pathways (19). Among its identified roles, AMPK has been implicated in the control of hepatic glucose and lipid homeostasis by many additional effects both on genes and on short-term regulation of specific enzymes. AMPK phosphorylates multiple targets in the liver in order to acutely switch on alternative catabolic pathways and switch off anabolic pathways. ACC and HMGCR were the first enzymes shown to be downstream targets for AMPK (20). ACC is an important rate-controlling enzyme for the synthesis of malonyl-CoA, which is both a critical precursor in the biosynthesis of fatty acids and a potent inhibitor of mitochondrial fatty acid oxidation. Inhibition of ACC by AMPK through phosphorylation leads to a fall in malonyl-CoA content and a subsequent decrease in fatty acid synthesis and increase in mitochondrial fatty acid oxidation via the allosteric regulation of carnitine palmitoyltransferase-1 (CPT-1), which catalyses the entry of long-chain fatty acyl-CoA into mitochondria.

Next, to confirm whether FG activates AMPK we attempted to inhibit the phosphorylations of AMPK and ACC by pharmacological approach. Pretreatment of HepG2 cells with compound C, an AMPK inhibitor (21), significantly attenuated phosphorylations of AMPK and ACC in a dose dependent manner (Fig. 3D). This result suggests that AMPK activation is required for the phosphorylation of ACC. Thus, the activation of AMPK by FG inhibits ACC, decreases malonyl-CoA levels, and leads to stimulation of fatty acid oxidation.

The discovery of AMPK kinases came from the identification of upstream kinases for the sucrose non-fermenting 1 (SNF1) complex, the yeast orthologue of AMPK. The nearest relative kinases in mammals were LKB1 and calmodulin-dependent protein kinase kinase β (22, 23). The major AMPK kinase activity in the liver seems to correspond to LKB1 since it has been shown that deletion of LKB1 in the liver results in a proportional decrease of AMPK phosphorylation rendering AMPK insensitive to stimuli which normally activate it (24). We also tested whether FG phosphorylates LKB1 in HepG2 cells. FG phosphorylated LKB1 in a time dependent manner (Fig. 3A), and phosphorylations of LKB1, AMPK, and ACC were occurred in a sequence (Fig. 3B).

**In vitro study/Effects of FG on triglyceride (TG) accumulation and related genes expression**

It has been reported that the plasma free fatty acid (FFA) elevation and the heterotrophic deposition of TG in the liver and skeletal muscle tissues are closely related to the development of insulin resistance (25). Viollet et al. (19) evidently reported that activation of AMPK in the liver leads to the stimulation of fatty acid oxidation and inhibition of lipogenesis (26). Therefore, we investigated the effect of FG on the expression of genes involved in lipogenesis and lipolysis. As shown in Fig. 4A, FG attenuated gene expression of sterol regulatory element-binding protein 1c (SREBP1c), central to the intracellular surveillance of lipid catabolism and de novo biogenesis, in a time dependent manner. Genes for FAS and SCD1, as well known target molecules of SREBP1c, were also marginally suppressed in a time dependent manner. In contrast, gene expression of PPAR-α, responsible for inducing lipid uptake and catabolism, was increased in a time dependent manner. Due to favorable effects on lipogenic and lipolytic gene expressions, FG was expected to reduce TG accumulation in the liver. As shown in Fig. 4B, TG accumulation was decreased by FG in a dose dependent manner. Compared to the basal level, fat storage was reduced to 20% at 500 µg/mL of FG. Together, these data strongly suggest that FG attenuates TG accumulation through regulation of gene expression involved in lipogenesis and lipolysis.
**In vivo** study/Effects of FG on metabolic parameters

Table 1 shows the effects of FG on metabolic parameters in diabetic db/db mice treated for 10 weeks. When the fasting plasma glucose levels were measured at the end of the experiment, all treatment groups [FG; 9.3 ± 0.6 mM (p < 0.001), FGH; 11.3 ± 0.9 mM (p < 0.01), MET; 4.9 ± 0.5 mM (p < 0.001)] showed significantly decreased plasma glucose levels, when compared to the DC group (15.8 ± 0.9 mM). The plasma insulin level was also detected. Compared to 295.7 ± 21.1 µU/mL in DC group, the plasma insulin concentrations of FGL, FGH, MET were 356.7 ± 27.9 µU/mL, 386.8 ± 17.7 µU/mL (p < 0.05), 343.0 ± 37.0 µU/mL, respectively. With the plasma glucose and insulin levels in each group, insulin resistance index was calculated using the equation described in Materials and Methods. Compared to the DC group, insulin resistance index (HOMA-IR) of the FG-treated groups (FGL, FGH) were significantly decreased by 33 and 12% (p < 0.05), respectively. HbA1c levels were also decreased by 1.2% (p < 0.01) in the FGL group and 0.8% (p < 0.01) in FGH group compared to that of DC group. Although there were no significant differences in plasma adiponectin levels between DC and FG-treated groups, plasma leptin levels of FG-treated groups were increased by 7% when compared to the DC group. In the meantime, there were no differences in plasma TG and non-esterified fatty acid (NEFA) level between DC and FG-treated groups.

**In vivo** study/Effects of FG on protein and gene expressions in liver and skeletal muscle

FG stimulates the phosphorylations of AMPK in liver (Fig. 5A) and skeletal muscle (Fig. 5B). GLUT4 protein expression in skeletal muscle was also enhanced in FGL and FGH treated groups. Next, we investigated the effect of FG on the expression of genes associated with lipogenesis and lipolysis in liver. As shown in Fig. 5C, gene expressions of SREBP1a, SCD1, and FAS were reduced in FG-treated groups, when compared to DC. In contrast, gene expressions

![Fig. 5. Effects of FG on AMPK expression in liver (A) and skeletal muscle (B), and lipogenic (C) and lipolytic (D) genes expressions in liver of db/db mice.](image-url)
of CD36 and PPAR-α were increased in FG-treated groups, when compared to DC (Fig. 5D). Together, these data strongly suggest that FG lowered plasma glucose levels through ameliorating insulin resistance and enhancing a glucose uptake into the skeletal muscle of db/db mice. 

Taken together, the relationship between AMPK activation and beneficial metabolic effects in db/db mice has provided the rationale for the development of new functional food or therapeutic agent based on nutritional or pharmacological use of FG in order to prevent or reverse hepatic disorders linked to type 2 diabetes and obesity.

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References