Ginsenoside Rg₁ Suppresses Hepatic Glucose Production *via* AMP-Activated Protein Kinase in HepG2 Cells

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Panax ginseng is known to have anti-diabetic activity, but the active ingredients are not yet fully identified. In this study, we found the inhibitory effect of Rg₁ on hepatic glucose production through AMP-activated protein kinase (AMPK) activation in HepG2 cells. Rg₁ significantly inhibited hepatic glucose production in a concentration-dependent manner, and this effect was reversed in the presence of compound C, a selective AMPK inhibitor. In addition, Rg₁ markedly induced the phosphorylations of liver kinase B1 (LKB1), AMPK and forkhead box class O1 (FoxO1), a key transcription factor for gluconeogenic enzymes, in time- and concentration-dependent manners. Glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) activities were inhibited by 24% and 21%, respectively, when the cells were exposed to 40 μ M of Rg₁, resulting from phosphorylation of FoxO1 and inhibition of gluconeogenic gene expression. Taken together, our results demonstrated the suppressive effect of Rg₁ on hepatic glucose production *via* LKB1-AMPK-FoxO1 pathway in HepG2 human hepatoma cells.

Key words Rg₁; AMP-activated protein kinase; hepatic glucose production; HepG2 cell

Type 2 diabetes mellitus (T2DM) is one of the devastating endocrine diseases in developed countries.¹⁾ The pathological change in glucose production of the liver is a central characteristic in T2DM.²⁾ Prolonged elevation of blood glucose levels can lead to many diabetic complications, such as cardiovascular disease, stroke, diabetic neuropathy, *etc.*¹⁾ Thus, the discovery of anti-diabetic agents inhibiting a hepatic glucose production is a focus of research for the pharmaceutical community.

AMPK-activated protein kinase (AMPK) is a heterotrimeric serine/threonine kinase that is widely expressed in a variety of organs including the liver. AMPK is considered as a master switch regulating glucose and lipid metabolism.³⁾ In the liver, activation of AMPK results in enhanced fatty acid oxidation and decreased productions of glucose, cholesterol, and triglycerides.⁴⁾ Gluconeogenic enzymes such as glucose-6-phosphatase (G6Pase) and phosphoenolpyruvate carboxykinase (PEPCK) are the rate-limiting enzymes in hepatic gluconeogenesis. Activation of AMPK suppresses G6Pase and PEPCK gene expressions and then decreases hepatic glucose production.⁵⁾ Therefore, AMPK might be an attractive target for therapeutic intervention in metabolic disorder such as T2DM and the metabolic syndrome.

Panax ginseng is a widely used herbal medicine in East

Asia and is reported to have anti-diabetic effects.^{6–13)} The active components of ginseng are thought to be ginsenosides, a group of steroidal saponin. Ginsenosides are distributed in many parts of the ginseng plant, including the root, leaf, and berry.¹⁴⁾ Rg₁ (Fig. 1A) is one of the pharmacologically active ginsenosides even though it is found only in trace amounts.¹⁵⁾ Numerous studies have demonstrated that Rg₁ can improve acquisition, assist in consolidation, retrieval of memory impaired by amnesic agents, increase the protein synthesis and cAMP level in the brain.^{16,17)} However, there are no reports demonstrating that Rg₁ might have an anti-diabetic activity by suppressing hepatic glucose production *via* AMPK activation. In the present study, we examined whether Rg₁ suppresses hepatic glucose production and this effect is associated with AMPK pathway in insulin-resistant HepG2 hepatoma cells.

MATERIALS AND METHODS

Chemicals Rg₁ was obtained from the Advanced Medica Biologica/Botanica Organics Institute (AMBO, Seoul, Korea), and dissolved in 0.1% dimethyl sulfoxide (DMSO). Antibodies against AMPK, acetyl-CoA carboxylase (ACC), phospho-AMPK, phospho-ACC, phospho-liver kinase B1

Fig. 1. Effect of Rg₁ on Hepatic Glucose Production

Chemical structure of ginsenoside Rg₁ (A). HepG2 cells were pretreated with Rg₁ or 100 nM of insulin for 6 h, incubated for 3 h in glucose production assay media, and hepatic glucose production was measured (B). * $p < 0.05$ vs. control.

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(LKB1), phospho-forkhead box class O1 (FoxO1) were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.), and anti-Actin from Santa Cruz Biotechnology (Santa Cruz, CA, U.S.A.). Compound C was obtained from Calbiochem (Darmstadt, Germany).

Cell Culture and Glucose Production Assay The HepG2 human hepatoma cell line was purchased from Korean Cell Line Bank (Seoul, Korea). HepG2 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) (GibcoBL, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum, 100 unit/ml penicillin, and 100 μ g/ml streptomycin in an atmosphere of 95% air and 5% CO₂ at 37 °C. Cells were cultured on 12-well plates at a density of 2×10^5 cells/well, and after 24 h cells were treated with or without Rg₁ in serum-free medium for 6 h. Cells were washed twice with phosphate buffered saline (PBS) to remove glucose and then incubated for 3 h in glucose production assay medium (glucose- and phenol red-free DMEM containing 2 mM sodium pyruvate, 20 mM sodium lactate). Fifty microliters of media solution were used for measuring the glucose concentration using an Amplex Red Glucose/Glucose Oxidase Assay Kit (Invitrogen, CA, U.S.A.). Glucose concentrations were normalized to cellular protein concentration measured by a Bio-Rad protein assay kit (Hercules, CA, U.S.A.).¹⁸⁾

Western Blot Analysis Equal amounts of protein (50 μ g/lane) were resolved by 8% sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoreses (SDS-PAGE) and transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, U.S.A.). The membrane was further incubated with specific antibodies such as p-AMPK, AMPK, p-ACC, ACC, p-LKB1, p-FoxO1 and Actin. The membrane was continuously incubated with appropriate secondary antibodies coupled to horseradish peroxidase and developed in the enhanced chemiluminescence (ECL) Western detection reagents. The immunoreactive bands were visualized and quantified by a densitometric analysis.

Determination of Enzyme Activity G6Pase activity was measured as follows: HepG2 cells were sonicated in ice-cold homogenization buffer (20 mM Tris-HCl, 5 mM ethylenediaminetetraacetic acid (EDTA), 250 mM sucrose, 10 μ M protease inhibitor, pH 7.0), followed by centrifugation at 12000 *g* for 20 min at 4 °C. The supernatant was subsequently centrifuged at 105000 *g* for 60 min at 4 °C. The pellet containing the microsomal proteins was resuspended in homogenization buffer, and the microsomal suspension was incubated with 20 mM glucose-6-phosphate and 50 mM Tris-cacodylate buffer (pH 6.5) in a final volume of 200 μ l for 20 min at 35 °C. The reaction was stopped by adding 200 μ l of 0.36 mM ammonium molybdate, 10% SDS, 1 mM H₂SO₄ and 1.4% ascorbic acid. After incubation for 30 min at 45 °C, the optical density at 820 nm was measured.¹⁹⁾ PEPCK activity was measured as follows: cells were sonicated in 1 ml of lysis buffer (10 mM Tris-HCl, 1 mM EDTA, 0.25 M sucrose, and 50 mM KCl, pH 7.2) and centrifuged at 10000 *g* for 30 min at 4 °C. After protein concentration determination, cells containing 100 μ g protein were added to the reaction buffer (50 mM Tris-HCl, 2 mM MnCl₂, 2.5 units/ml malate dehydrogenase, and 0.15 mM reduced nicotinamide adenine dinucleotide) in a 96-well plate. The reaction was initiated with the addition of 0.4 mM deoxy-guanosine 5'-diphosphate

and the decrease in absorbance at 340 nm was measured for 5 min.²⁰⁾

Statistical Analysis All data were expressed as a mean $\pm$ standard error (S.E.). Comparisons between groups were analyzed using Student's *t*-test, and considered significantly different when *p* < 0.05.

RESULTS AND DISCUSSION

The regulation of hepatic glucose production is an important process in the adjustment of the blood glucose level, and abnormal changes in the glucose production of the liver are a chief characteristic in type 2 diabetes. The rate of hepatic glucose production in type 2 diabetic patients is considerably increased compared with healthy subjects, thereby contributing significantly to the fasting hyperglycemia.²⁾

For more than 2000 years, traditional Chinese medicine has used the *Panax ginseng* for a variety of diseases, including symptoms similar to those of T2DM.⁶⁾ A number of active components of *Panax ginseng* have been isolated and characterized; among these, Rg₁ is one of the active components of protopanaxatriol ginsenosides. Couple of reports demonstrating anti-diabetic activities of Rg₁,^{21,22)} and pivotal roles of AMPK in hepatic glucose metabolism gave us an opportunity to examine the hypothesis that Rg₁ might suppress hepatic glucose production *via* activating AMPK in HepG2 cells.

To examine cytotoxicity of Rg₁, various concentrations of Rg₁ (from 5 to 80 μ M) were treated on HepG2 cells for 24 h. Rg₁ did not show any cellular toxicity up to 80 μ M (data not shown). Thereafter, we employed less than 80 μ M of Rg₁ in subsequent experiments.

To examine the influence of Rg₁ on hepatic glucose metabolism, we measured glucose production in HepG2 cells. HepG2 cells were treated with indicated concentrations of Rg₁ for 6 h. Rg₁ inhibited the hepatic glucose production in a concentration-dependent manner. After 6 h exposure to 40 μ M of Rg₁, hepatic glucose production was significantly inhibited by 22%, comparable to insulin-treated cells (Fig. 1B).

LKB1 is a one of AMPK kinase in the liver and responsible for phosphorylation and activation of AMPK. The effect of metformin on AMPK activation and glucose lowering efficacy was abolished in LKB1^{-/-} mice.²³⁾ The effects of AMPK activation are pleiotropic in metabolically relevant tissues, such as liver, skeletal muscle, adipose, and hypothalamus. In the liver, AMPK regulates glucose homeostasis mainly through the inhibition of gluconeogenic gene expression and hepatic glucose production.²⁴⁾

As shown in Fig. 2, Rg₁ significantly stimulated the phosphorylation of AMPK and ACC, an immediate substrate of AMPK, in time- and concentration-dependent manners. LKB1 was also phosphorylated at Thr189 in time- and concentration-dependent manners (panels A and B of Fig. 2). Akt is one of the two kinases known to suppress hepatic glucose production. However, Rg₁ did not stimulate phosphorylations of Akt and phosphoinositide-3 kinase (PI3K) (data not shown). Next, to determine whether AMPK plays a role in the Rg₁-mediated reduction of hepatic glucose production, we used a selective AMPK inhibitor, compound C. HepG2 cells were pretreated with 20 μ M of compound C for 1 h, and

then incubated with 40 μM of Rg_1 for 6 h. AMPK activation and reduction of hepatic glucose production caused by Rg_1 were completely blunted in the presence of compound C (panels C and D of Fig. 2).

The principal parameters affecting hepatic glucose output are the concentrations of the available gluconeogenic sub-

strates and the activity of a few regulatory enzymes. As shown in Fig. 3, G6Pase and PEPCK activities were inhibited by 24% and 21%, respectively, when the cells were exposed to 40 μM of Rg_1 . Next, we examined the effect of Rg_1 on transcription factor to clarify the underlying mechanism. Gene expression of the key gluconeogenic enzymes is regu-

Fig. 2. Effects of Rg_1 on LKB1-AMPK-FoxO1 Cascade

HepG2 cells were treated with 40 μM of Rg_1 for the indicated times (A), and also treated with the indicated concentrations of Rg_1 for 6 h (B). HepG2 cells were pretreated with compound C for 30 min, and then treated with Rg_1 for 6 h (C). In the same condition, glucose levels in media were measured as described under Materials and Methods (D). ** $p < 0.01$ vs. control.

Fig. 3. Effect of Rg_1 on Activities of Gluconeogenic Enzymes

HepG2 cells were pretreated with Rg_1 or 100 nM of insulin for 6 h in serum-free media. The G6pase and PEPCK activities were measured as described under Materials and Methods and these activities were compared between groups as shown in panels A and B of the figure, respectively. Values represent the means and standard error for three independent experiments. * $p < 0.05$, ** $p < 0.01$ vs. control.

lated by transcriptional and non transcriptional mechanisms.²⁾ Interestingly, AMPK α 2 containing complexes have been identified in the nucleus, consistent with a role in gene regulation. In addition, recent studies suggest that AMPK may directly or indirectly regulate transcriptional activators such as TORC2 (transducer of regulated cyclic AMP (cAMP) response element binding protein activity 2) and FoxO1 to control liver glucose metabolism.²⁵⁾ 5-Aminoimidazole-4-carboxamide riboside and glucose starvation inhibits G6Pase expression by reduction of the cellular level of FoxO1, presumably mediated by specific degradation of the protein.²⁶⁾ Also prolonged shear stress led to activation of AMPK and the subsequent phosphorylation and degradation of FoxO1.²⁷⁾ As shown in panels A and B of Fig. 2, Rg₁ phosphorylated FoxO1 in time- and concentration-dependent manners. These results may explain how Rg₁ inhibits G6Pase and PEPCK enzyme activities.

In summary, Rg₁ suppressed the hepatic glucose production by stimulating AMPK, and LKB1-AMPK-FoxO1 signaling module may be responsible for this pharmacological effect. Providing with *in vivo* anti-diabetic activity of Rg₁ in the future, Rg₁ has a potential to treat type 2 diabetic patients.

Acknowledgements This work was supported by a Grant from the Graduate Research Scholarship of Kyung Hee University Graduate School and a Grant (M106KD010018-09K0401-01810) from the Plant Diversity Research Center of the 21st Century Frontier Program.

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