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Geniposide Suppresses Hepatic Glucose Production via AMPK in HepG2 Cells

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Geniposide is one of the main compounds in Gardenia jasminoides Ellis and has many pharmacological activities, but its anti-hyperglycemic activity has not yet been fully explored. This study was designed to determine, for the first time, how geniposide from G. jasminoides regulates hepatic glucose production, and the underlying mechanisms. During an in vitro study, we found the inhibitory effect of geniposide on the hepatic glucose production is partly through AMP-activated protein kinase (AMPK) activation in HepG2 cells. Geniposide significantly inhibited hepatic glucose production in a dose-dependent manner. AMPK, acetyl coenzyme A synthetase (ACC) and forkhead box class O1 (FoxO1) phosphorylation were stimulated by different concentrations of geniposide. In addition, the enzyme activities of phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase) were all significantly suppressed. What is important is that these effects were partly reversed by (1) inhibition of AMPK activity by compound C, a selective AMPK inhibitor, and by (2) suppression of AMPKα expression by small interfering RNA (siRNA). In summary, geniposide potentially ameliorates hyperglycemia through inhibition of hepatic gluconeogenesis by modulation of the AMPK–FoxO1 signaling pathway. Geniposide or geniposide-containing medicinal plants could represent a promising therapeutic agent to prevent type 2 diabetes on gluconeogenesis.

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

Type 2 diabetes mellitus (T2DM) comprises heterogeneous disorders that result in chronic hyperglycemia and life-threatening complications. The pathological change in hepatic glucose production is a central characteristic in diabetic patients.1) Hyperglycemia in both types 1 and 2 diabetes mellitus is associated with high hepatic glucose production.2) Suppression of hepatic glucose production has been shown to improve overall glycemic control in both human patients and type 2 diabetes animal models.3) The control of hepatic glucose production depends on many factors, including the key gluconeogenic enzymes phosphoenolpyruvate carboxykinase (PEPCK) and glucose-6-phosphatase (G6Pase), insulin levels, and the concentration of gluconeogenic substrates (lactate, gluconeogenic amino acids, and glycerol). A variety of molecular signaling pathways were demonstrated to regulate hepatic glucose production, such as AMP-activated protein kinase (AMPK) activation,4) protein kinase B (AKT or PKB) phosphorylation and mitogen-activated protein kinase (MAPK) inhibition. Among them, AMPK, the evolutionarily conserved serine/threonine kinase, is considered as the major signaling molecule responsible for this process. AMPK acts as an intracellular sensor to maintain the glucose homeostasis in the hypothalamus, skeletal muscle and liver5–7) by the phosphorylation of several downstream metabolic enzymes and certain transcription factors. In the liver, the acute activation of AMPK, achieved by adenovirus-mediated gene transfer of a constitutively active form of AMPKα2, is sufficient to decrease blood glucose levels and reduce hepatic gluconeogenic gene expression, such as PEPCK and G6Pase.8) Work with pharmacological compounds and adenovirus-mediated AMPK activation/inactivation strategies suggested that hepatic AMPK pathway might be an attractive drug target for metabolism regulation.9)

Geniposide, a major iridoid glycoside found in Gardenia jasminoides Ellis fruits, is widely used in Asian countries.9) It has been reported to have a wide spectrum of biological and pharmacological activities, including hepatoprotection,10) neuroprotection,11,12) anti-inflammation,13) anti-nociception,14) anti-diabetes,15,16) anti-apoptosis17) and anti-allergic.18) The first report of geniposide hypoglycemic activity in high sugar diet-induced diabetic mice was made in 1982.19) Some studies further confirmed the hypoglycemic effects of geniposide in diabetic mice induced by a high-fat diet and streptozotocin.20) However, little is known about the biochemical mechanisms by which geniposide regulates hepatic glucose production. We previously reported that geniposide improves blood sugar control in db/db mice and increases acute insulin secretion in response to low and moderately high glucose levels through modulating glucose metabolism and tricarboxylic acid cycle (TCA) in insulin-secreting (INS)-1E β cell line.15,16) AMPK is the key enzyme involved in this regulation (data not shown). Based on the overlap in the metabolic pharmacology of AMPK and our previous results, we supposed that geniposide might regulate hepatic glucose production through adjusting the key regulatory enzymes in glucose metabolism, and AMPK signaling pathway plays an important role in this process. In this context, we evaluated underlying mechanisms of geniposide’s effects on glycolysis and glucose consumption with emphasis on the roles of AMPK in HepG2 cells.

MATERIALS AND METHODS

Chemicals  Geniposide was obtained from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), and dissolved in 0.1% dimethyl sulfoxide (DMSO). Compound

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C (6-{[4-(2-piperidin-1-yletoxy)-phenyl]-3-pyridin-4-ylpyrazolo[1,5-a]pyrimidine} was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.). Cell proliferation assay kit was from Promega (Mannheim, Germany). Specific antibodies against phospho-AMPK, AMPKα1/2, phosphor-aceetyl coenzyme A synthetase (ACC), ACC, phosphor-forked box class OI (FoxO1), FoxO1 were purchased from Cell Signaling Technology (Beverly, MA, U.S.A.). All other reagents not mentioned were of the highest grade commercially available.

**Cell Culture and Treatments** The HepG2 human hepatoma cell line was purchased from CCTCC (China Center for Type Culture Collection). The cells were cultured in Dulbecco’s modified Eagle’s medium (DMEM) (Gibco BRL, Grand Island, NY, U.S.A.) containing 10% fetal bovine serum (FBS), 100 unit/mL penicillin, and 100 µg/mL streptomycin in an atmosphere of 95% air and 5% CO2 at 37°C. One day after plating the medium was changed to DMEM containing 5.5 mM D-glucose, 2 mM glutamine and 10% FBS, and the culture was continued. Subsequently, the experimental treatment was carried out for the indicated periods with various concentrations of geniposide in serum-free media containing 5.5 mM D-glucose 2 mM glutamine.

Cells were treated with or without different concentrations of geniposide in serum-free medium for 6 h. In the experiments with the pharmacological inhibitors, cells were preincubated with 20 µM compound C for 30 min prior to 6 h of geniposide treatment.

**Determination of Cell Proliferation and Cytotoxicity** Cells (5 × 104 cells/well) were plated in 100 µL of cell culture medium in a 96-well plate. After different periods of incubation (24 and 72 h), cell proliferation was determined using the CellTiter 96® AQueous Non-Radioactive Cell Proliferation Assay (MTS; Promega, Mannheim, Germany) according to the manufacturer’s instructions. The absorbance of each well was measured using a microplate reader at 490 nm. For measuring cytotoxicity, the LDH Plus Cytotoxicity Kit (Roche) was used according to the manufacturer’s instructions.

**Glucose Production Assay** HepG2 cells were seeded in 24-well plates (2 × 105 cells/well) and treated with or without geniposide 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). Media solution was used for measuring the glucose concentration using a colorimetric glucose assay kit (Sigma). The readings were then normalized to the total protein content determined from the whole-cell lysates.

**Immunoblotting** Western blot analysis was performed using whole cell lysates. The HepG2 cells were washed with cold PBS and lysed in a lysis buffer containing 20 mM Tris–HCl (pH 7.5), 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid (EDTA), 1% (v/v) Triton X-100, 0.1% sodium dodecyl sulfate (SDS), protease inhibitors (aprotinin, 30 µg/mL; leupeptin, 4 µg/mL; pepstatin, 2 µg/mL; and phenylmethyl sulfonyl fluoride, 10 µg/mL), 1 mM NaN3, and 2.5 mM Na2P2O7. Lysates were sonicated and measured for protein concentration. The samples were stored at −80°C for other analyses. An aliquot of 10–20 µg protein from each cell extract was loaded on a 10% SDS-polyacrylamide gel electrophoresis (PAGE) gel. After electrophoretic separation, proteins were transferred to polyvinylidene difluoride (PVDF) membrane. Primary and secondary antibodies were diluted in a blocking solution and incubated with the membrane for indicated times. Excess antibody was washed off with 20 mM Tris-buffered saline containing Tween-20 (TBST, 20 mM Tris, 150 mM NaCl and 0.1% Tween 20; pH 7.5). Immunoreactivity was detected using enhanced chemiluminescence (ECL) Western blotting kit (Amersham Pharmacia Biotech AB, Uppsala, Sweden). Bands were analyzed by densitometric scanning using the Quantity One software (BioRad, Munich, Germany).

**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 EDTA, 250 mM sucrose, 10 µM 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 µL for 20 min at 35°C. The reaction was stopped by adding 200 µL 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.21)

**PEPCK activity** was measured as follows: cells were sonicated in 1 mL of lysis buffer (10 mM Tris–HCl, 1 mM EDTA, 0.25 mM 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 µg 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.22)

**Small Interfering RNA (siRNA) Transient Transfection** siRNA oligonucleotides targeting AMPK (Homo sapiens) were purchased from Santa Cruz Biotechnology (Catalog# sc-29673, Santa Cruz, CA, U.S.A.). A scrambled siRNA was used as a control. In brief, one day before transfection, HepG2 cells were placed into 6-well plates for 24 h. And cells were transfected with 30 pmol/well siRNA via Lipofectamine 2000 Transfection Reagent (Invitrogen, Carlsbad, CA, U.S.A.) per the manufacturer’s directions. To rule out the possibility of siRNA off-targeting, experiments were repeated with 2 other siRNAs (Shenggong, Shanghai, China) targeting homo sapiens AMPKα1 (sense: gcggagcgaaauggauaTT; antisense: uaaucuauaguccucucTT) and AMPKα2 (sense: gcgguuugg-uguagguasaTT; antisense: uacauccacaaacacggTT). All experiments were repeated with individual siRNAs for 3 times independently and the same results were reached.

**Statistical Analysis** All data are expressed as the mean ± standard deviation (S.D.). Statistical analyses were conducted using the software of Origin version 8.0 (OriginLab Corporation, MA, U.S.A.). Data were analyzed using one-way ANOVA followed by the Tukey’s post-hoc test for the differences among the treatment, where p<0.05 was considered significant.

**RESULTS**

**Geniposide Had No Cytotoxicity on HepG2 Cells** To determine the potential effects of geniposide on cell viability.
ity and cytotoxicity of HepG2, cells were exposed to geniposide treatment at different concentrations (0–200 µM) for 24 or 72 h. Lactate dehydrogenase (LDH) cytotoxicity assay and 3-(4,5-Dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) cell viability assay were conducted to address these issues. Treatment of HepG2 cells for 24 or 72 h with geniposide did not induce changes in cell viability or LDH release, indicating that geniposide did not damage cell integrity even at a highest concentration of 200 µM (Table 1). Thereafter, geniposide of 0–100 µM was used in the subsequent experiments, a range of concentration consistent with that used in previous studies.15,16,20

### Geniposide Improved Glucose Consumption in Vitro
To identify the effect of geniposide on hepatic glyc metabolism, glucose production was examined in HepG2 cells after treatment with geniposide for 6 h. As illustrated in Fig. 3A, compound C fully blocked geniposide-induced ACC and FoxO1 phosphorylation, and decreased the phosphorylation level of AMPK. With the treatment of compound C, the inhibition of hepatic glucose production induced by geniposide was markedly prevented (Fig. 3B), and geniposide decreased the activity of PEPCk, and the blockage of AMPK induced a significant increase in the G6Pase activity (p<0.01) in HepG2 cells (Figs. 2B, 3B).

### Geniposide-Stimulated Glucose Consumption Was Reduced upon AMPKα1/2 Silencing in HepG2 Cells
To further prove the effect of AMPK on glucose-lowering action of geniposide, AMPKα1/2 siRNA was used to inhibit the expression, and activation of AMPKα in HepG2 cells. As shown in Fig. 4, compared with scrambled siRNA, AMPKα1/2 siRNA at 40 pmol/well significantly decreased the expression of AMPKα (p<0.01), and the expression of AMPKα was most effectively suppressed by 70% by AMPKα1/2 siRNA at 40 pmol/well, meanwhile inhibition of geniposide-induced hepatic glucose production, PEPCk and G6Pase activities were markedly prevented by 40 pmol/well AMPKα siRNA. Meanwhile, experiments with 2 other siRNAs against 2 other different AMPKα1/2’s sequences showed a similar result, which suggested that such an effect of geniposide was real and not a result off-target knockdown (Fig. 4). It was similar to compound C study.

### DISCUSSION
In the late 2000s, as a result of screening novel glucagon-like peptide-1 receptor (GLP-1R) agonists from active components of traditional Chinese herbs,24 we found geniposide, with the activation of GLP-1R, induced insulin secretion in a dose-dependent manner and enhanced glucose-stimulated insulin secretion (GSIS) in INS-1 cells in a phosphatidylinositol 3-kinase (PI3K) dependent mechanism.15,25 Further work-up showed that geniposide improves blood sugar control in db/db mice and increases acute insulin secretion in response to low and moderately high glucose levels through modulating glu-
cose metabolism,\textsuperscript{15,16} and AMPK, a pivotal molecule in cellular metabolism, is the key enzyme involved in this regulation (data not shown).

In T2DM, the liver determines the increase in fasting plasma glucose concentrations that results from greater basal endogenous glucose production\textsuperscript{26} due to the elevated gluconeogenesis\textsuperscript{27} and despite higher fasting insulinemia.\textsuperscript{28} One of the most efficient ways to modulate glucose metabolism in diabetic patients would be a perturbation on hepatic glucose production. Because the liver plays an important part in maintaining glucose homeostasis \textit{via} glycogenolysis and gluconeogenesis, we speculated that the modulation of glucose metabolism by geniposide in the liver may be involved in the hypoglycemic mechanism of geniposide. Recently, it was found that geniposide significantly decreased the blood glucose and triglyceride (TG) levels in diabetic mice in a dose-dependent manner by inhibiting the glycogen phosphorylase (GP) and G6Pase activities at least in part.\textsuperscript{20} But the most direct connection between the glycemic control and the hepatic glucose output and the in-depth mechanism of action of geniposide are not quite sure. We examined whether geniposide treatment inhibits hepatic endogenous glucose production in a hepatic cell line of human origin (HepG2). The results in the present study demonstrated for the first time that geniposide, at least in part, inhibited the hepatic glucose production in a dose-dependent manner with the activation of AMPK pathway.

AMPK is a phylogenetically conserved serine/threonine protein kinase that has been proposed to act as a ‘metabolic master switch’ mediating the cellular adaptation to environmental or nutritional stress factors. In situations of energy consumption, a decrease in the cellular ATP to AMP ratio leads to AMPK activation and inhibition of energy-consuming biosynthetic pathways, such as fatty acid and sterol synthesis, and activation of ATP-producing catabolic pathways, such as fatty acid oxidation.\textsuperscript{29} Work with pharmacological compounds and adenovirus-mediated AMPK activation/inactivation strategies suggest that hepatic AMPK has a regulatory role in glucose homeostasis by regulating specific enzymes.\textsuperscript{30} As shown in Fig. 2, geniposide significantly stimulated the phosphorylation of AMPK and ACC, an immediate substrate of AMPK.

![Fig. 2. Effects of Geniposide on AMPK–FoxO1 Cascade and the Activities of Gluconeogenic Enzymes in HepG2 Cells](image)

HepG2 cells were treated with the indicated concentrations of geniposide for 6h. (A) Whole cell lysate protein was used to measure the phosphorylation of AMPK (p-AMPK), ACC (p-ACC) and FoxO1 (p-FoxO1) by Western blot in the HepG2 cells. Percentage values of p-AMPK/AMPK, p-ACC/ACC and p-FoxO1/FoxO1 ratio relative to the control condition (mean±S.D., n=5). *p<0.05 and **p<0.01 vs. control in corresponding group. (B) The G6Pase and PEPCK activities were measured as described under Materials and Methods and these activities were compared between groups as shown in panels B of the figure, respectively. Data are expressed as the mean±S.D. from three representative experiments. *p<0.05 and **p<0.01 vs. control.
by using compound C of AMPK inhibitor and AMPKα1/α2 siRNA. AMPK activation and reduction of hepatic glucose production caused by geniposide were partly blunted in the presence of compound C (Fig. 3) or AMPK siRNA (Fig. 4).

Gluconeogenic enzymes such as PEPCK and G6Pase are the rate-limiting enzymes in hepatic gluconeogenesis. Activation of AMPK suppresses PEPCK and G6Pase gene expressions and then decreases hepatic glucose production.31 In the present work, geniposide inhibited PEPCK and G6Pase activities in HepG2 cells (Fig. 2), but in cells incubated with compound C or AMPK siRNA these effects were prevented with the reduction of hepatic glucose production (Figs. 3, 4).

In agreement, we have shown that AMPK is involved in the modulation of PEPCK, G6Pase activities and the production of glucose in HepG2 cells treated with geniposide.

Among the transcription factors that are critical in regulating gluconeogenic genes (e.g., PEPCK or G6Pase), FoxO1 is most tightly linked with the AMPK-dependent regulation of hepatic glucose metabolism.30,32 FoxO1, an important transcriptional regulator for energy metabolism in mammals, is shown to regulate this pathway in multiple insulin-sensitive tissues, such as adipose tissue and skeletal muscle.32,33 In the liver, FoxO1 is responsible for the activation of gluconeogenesis by the transcriptional activation of gluconeogenic
genes. As shown in Fig. 2, geniposide phosphorylated FoxO1 in dose-dependent manners. These results may explain how geniposide inhibits G6Pase and PEPCK enzyme activities.

In conclusion, the results of our study demonstrated that geniposide improves hepatic gluconeogenesis, suggesting that geniposide is the suppressors of hepatic gluconeogenic and glucolytic enzymes. The mechanism of gluconeogenic and glucolytic enzymes suppression by geniposide was associated with the activation of AMPK in HepG2 cells. New preparation including geniposide represents an attractive potential therapeutic strategy for the treatment of type 2 diabetes and other components of the metabolic syndrome.

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Fig. 4. Effects of Geniposide on Glucose Production and Gluconeogenic Enzymes upon AMPKα1/α2 Silencing in HepG2 Cells

(A) The interfering efficiency of siRNA for AMPKα1/α2 gene was determined by Western blot. AMPKα1/α2 siRNA at 40 pmol/well most effectively suppressed the expression of AMPKα. **p<0.01 vs. blank control. (B) Interference on AMPKα1/α2 expression reversed the influence of geniposide on glucose production. (C) Interference on AMPKα1/α2 expression inhibited the effect of geniposide on gluconeogenic enzymes. Data are expressed as the mean±S.D. from three representative experiments. **p<0.01 vs. control in corresponding group.
Conflict of Interest The authors declare no conflict of interest.

REFERENCES


