JOURNAL OF AGRICULTURAL AND FOOD CHEMISTRY

Compound K Enhances Insulin Secretion with Beneficial Metabolic Effects in *db*/*db* Mice

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Compound K (CK) is a final metabolite of panaxadiol ginsenosides. Although *Panax ginseng* is known to have antidiabetic activity, the active ingredient is not yet fully identified. In our preliminary studies, panaxadiol ginsenosides showed insulin secretion stimulating activity. Therefore, it would be interesting to know whether and how CK has antidiabetic activity. In in vitro studies using HIT-T15 cells and primary cultured islets, CK enhanced the insulin secretion in a concentration-dependent manner. This effect, however, was completely abolished in the presence of diazoxide (K^+ channel opener) or nifedipine (Ca^{2+} channel blocker). Insulin secretion stimulating activity of a single oral CK administration was also confirmed with an oral glucose tolerance test (OGTT) using ICR mice. From these studies, we may conclude that CK lowered the plasma glucose level by stimulating insulin secretion and this action was presumably associated with an ATP-sensitive K^+ channel. In a long-term study using C57BL/KsJ *db*/*db* mice, CK treatment significantly decreased the fasting blood glucose levels in a dose-dependent fashion. OGTT revealed that CK improved glucose tolerance with increased insulin levels 30 min after the glucose challenge. Concurrently, CK treatment prevented the destruction of islets and preserved more insulin. Next, to gain insight into the extra-pancreatic molecular mechanism of CK, we performed a global gene expression profiling study in the liver and adipose tissues. According to DNA microarray analysis, CK shifted glucose metabolism from hepatic glucose production to hepatic glucose utilization in the liver and improved insulin sensitivity through enhancing plasma adiponectin levels, resulting in overexpression of genes for adipogenesis and glucose transporter in the adipose tissue. Taken together, we may suggest that CK could be developed as a therapeutic tool in type 2 diabetic patients with disability of insulin secretion and/or insulin resistance.

KEYWORDS: Compound K; diabetes; ATP sensitive K^+ channel; oral glucose tolerance test; DNA microarray

INTRODUCTION

Diabetes mellitus is a serious metabolic disease affecting major populations worldwide. The number of people with diabetes is anticipated to rise from the current estimate of 150 million to 220 million in 2010 and 300 million in 2025 (*1*). High caloric diets and sedentary lifestyles in industrialized societies are fundamental causes of this fast-spread "epidemic". Diabetes mellitus is divided into two main forms. Type 1 diabetes mellitus (T1DM) is mainly due to an autoimmune-mediated destruction of pancreatic β-cell islets. On the other hand, type 2 diabetes mellitus (T2DM) is characterized by insufficient insulin secretion and insulin resistance (*2*). The insulin resistance provokes glucolipotoxicity in pancreatic β-cells, compensatory hyperinsulinemia, amyloid deposits, and inflammation in pancreatic islet (*3*). Consequently, the function of pancreas is gradually lost.

Epidemiological studies and clinical trials strongly support the notion that hyperglycemia is the principal cause of microvascular and macrovascular complications. Therefore, effective blood glucose control is the key to preventing or reversing diabetic complications and improving quality of life in diabetic patients (*4*, *5*). Although no cure is yet available for T2DM, oral hypoglycemic agents have been developed and are widely used. These therapies, however, are not perfect and characterized by insufficient efficacy, limited tolerability, or significant mechanism-based adverse effects. Therefore, novel treatment options are urgently needed that take advantage of

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10.1021/jf0722598 CCC: $37.00
Published on Web 11/23/2007

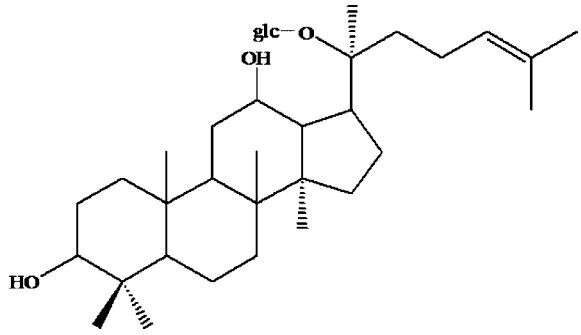

Figure 1. Chemical structure of compound K.

physiological regulatory mechanisms and that result in weight loss or lack of weight gain.

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 (*6*). There have been lots of studies demonstrating the antidiabetic activity of ginsenosides (*7–9*); however, the active component with antidiabetic activity is yet to be identified.

In the preliminary study, panaxadiol ginsenosides potentiated an insulin secretion stimulated by a low concentration of glucose. Compound K (CK), a final metabolite of panaxadiol ginsenosides (**Figure 1**), showed the most potent insulin secretion stimulating activity. It has been reported that CK has antitumor, apoptotic and hepatoprotective effects (*10–12*). However, the antidiabetic effect of CK has not been studied elsewhere as of this writing. Therefore, it would be interesting to examine whether and how CK has antidiabetic activity. To investigate antidiabetic activity of CK, the present study was divided into two parts. First, we examined the insulin secretion stimulating activity of CK using HIT-T15 cells and primary cultured islets, followed by the oral glucose tolerance test using ICR mice. Second, the antidiabetic activity and mechanism(s) of CK was elucidated in C57BL/KsJ *db*/*db* mice.

MATERIALS AND METHODS

Drugs and Chemicals. CK was obtained from the Central Research Center, ILHWA Pharmaceutical Co. (Guri, Korea). CK was dissolved in 0.1% DMSO (Sigma, St. Louis, MO) for in vitro study, and in 1% Tween 80 (Yakuri Pure Chemicals, Kyoto, Japan) for in vivo study. Diazoxide, nifedipine, and metformin were purchased from Sigma. Rat and mouse insulin enzyme immunoassay ELISA kits were bought from Shibayagi (Gunma, Japan). Other reagents were of the highest purity commercially available.

Animals. Our study was reviewed and approved by the Animal Care and Use Committee of Kyung Hee University. Six-week-old male ICR mice and C57BL/KsJ *db*/*db* mice were purchased from ORIENT BIO (Sungnam, Korea), and they were acclimatized for 2 week before being randomly assigned into the experimental groups. The animals were housed in a room with a 12–12 h light−dark cycle (8:00 a.m. to 8:00 p.m.), a temperature of 24 ± 1 °C, and a humidity of 55 ± 5%. During the acclimatization period, animals were fed standard rodent chow (LabDiet, Richmond, VA) and water ad libitum.

HIT-T15 Cells and Primary Islets Culture. Hamster pancreatic β-cell line, HIT-T15, was kindly obtained from Dr. K. S. Suh of the Kyung Hee Medical Center (Seoul, Korea). HIT-T15 cells (between passages 75–80) were cultured in RPMI 1640 media containing 11.1 mM glucose with 10% fetal bovine serum (FBS), 100 U/mL penicillin, and 100 μg/mL streptomycin. The medium was changed every 2 days, and cells were subcultured every 5–6 days.

Pancreatic islets were isolated from male Sprague–Dawley rats weighing 100–150 g. The rats were killed by cervical dislocation after anesthesia. The islets were isolated by injecting collagenase type V in Hanks' balanced salt solution (HBSS) (12 mg/12 mL per rat) into the pancreas through the pancreatic duct. The gland was removed, incubated for 10 min at 37 °C, and washed with HBSS solution, and islets were picked up after separation by Biocoll separating solution (Biochrom AG, Berlin, Germany). Isolated islets were cultured overnight free floating in a six-well plate in RPMI 1640 media with 11.1 mM glucose, 10% FBS, 100 U/mL penicillin, and 100 μg/mL streptomycin at 37 °C, atmosphere 5% CO_2/O_2. After incubation, five to six islets were transferred to 24-well plate and incubated for 7 days.

Insulin Secretion. HIT-T15 cells were seeded into a 24-well plate at a density of 2×10^5 cells per well and grown for 24 h. The cells were washed twice and preincubated for 30 min in Krebs-Ringers bicarbonate (KRB) buffer (115 mM NaCl, 4.7 mM KCl, 2.56 mM $CaCl_2$, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 20 mM $NaHCO_3$, 16 mM HEPES, and 0.3% bovine serum albumin, pH 7.4). Cells were then treated with KRB buffer containing 5 mM glucose with or without CK, and incubated for 1 h at 37 °C. After incubation, aliquots of the media were stored at −20 °C until insulin measurement. The same method was applied to the isolated islets except using 11.1 mM glucose. To explore how CK augments the glucose-stimulated insulin secretion, HIT-T15 cells were incubated for 1 h in KRB buffer containing either 0.5 mM diazoxide (K^+ channel opener) or 10 μM nifedipine (L-type of Ca^{2+} channel blocker) in the absence or presence of CK (8 μM), and insulin concentration was measured.

Oral Glucose Tolerance Test (OGTT) in ICR Mice. The ICR mice were allowed to fast for 12 h prior to the experiment, and CK (12.5 and 25 mg/kg of body weight) was administered orally 30 min prior to the glucose challenge. Glucose (1.5 g/kg) was orally administered at 0 min, and the blood was withdrawn from the orbital venous plexus at 0, 30, 60, and 120 min after glucose administration. Plasma glucose and insulin levels were determined by the glucose oxidase method (*13*) and mouse insulin ELISA kit, respectively.

Multiple administration of CK. *Treatment.* Eight week old, C57BL/KsJ *db*/*db* mice were randomly divided into four groups, the diabetic control group and three treatment groups. The diabetic control (CON) mice continued to receive standard rodent chow, and the treatment groups were fed standard rodent chow with either 10 or 20 mg/kg of body weight CK (CK10 and CK20) for a 25 day period. As a positive control, metformin was orally administered at a dose of 300 mg/kg (MT300). During the experiment, body weight and blood glucose levels were measured every week. OGTT was performed on the 22nd day. At the end of the study, blood was also collected for determinations of hemoglobin A1c (HbA1c), insulin, adiponectin, and lipid levels. Plasma triglyceride (TG), total cholesterol, and HDL cholesterol (HDL-C) were determined using commercially available kits (Asan Pharmaceutical Co., Seoul, Korea). Plasma nonesterified fatty acid (NEFA) and adiponectin levels were determined using an enzymatic colorimetric method (Eiken, Tokyo, Japan) and a mouse adiponectin ELISA kit (Adipogen, Seoul, Korea), respectively. HbA1c in whole blood was measured using a hemoglobin A1c kit (BioSystems S.A., Barcelona, Spain). After sacrifice, white adipose tissue and liver were immediately removed and instantly soaked in liquid nitrogen and stored at −70 °C for mRNA analyses and determination of enzyme activities.

Anti-Insulin Immunostaining. The pancreas was removed and fixed in 10% neutral buffered formalin. The tissues were subsequently embedded in paraffin and sectioned with thickness of 5 μm using a microtome (Leica, Wetzlar, Germany). The sections prepared onto aminosilane-treated slides were deparaffinized and rehydrated through graded alcohols to distilled water, and incubated with 0.1% trypsin and normal rabbit serum blocking solution for 30 min to block nonspecific binding of immunoglobulin. The sections were incubated with goat snti-insulin A (C-12) (Santa Cruz Biotechnology, Santa Cruz, CA) for 1 h at room temperature and treated with 3% hydrogen peroxide for 10 min to block endogenous peroxidase activity. The tissues were then incubated with biotinylated rabbit anti-goat IgG for 30 min at room temperature. The tissues were labeled using a modification of the avidin–biotin complex immunoperoxidase staining procedure (Vectastain Elite ABC kit, Vector Laboratories, Burlingame, USA). Positive staining was visualized using DAB peroxidase substrate solution for 5–10 min, and tissues were counterstained with hematoxylin.

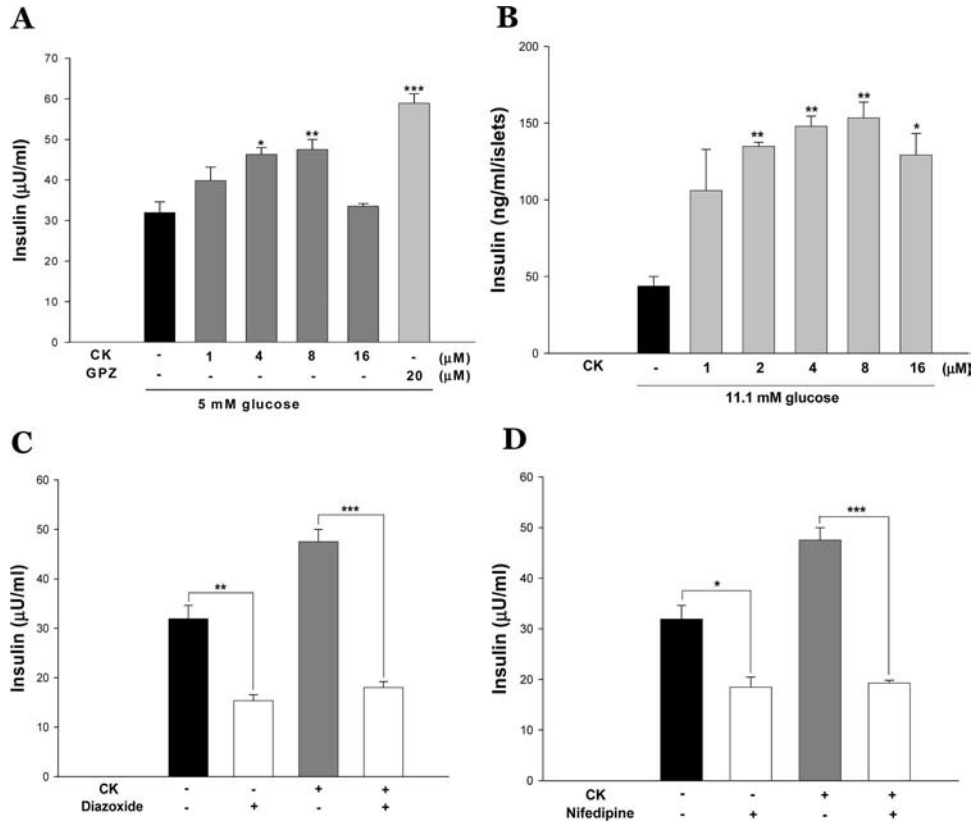

Figure 2. Effect of CK on glucose-stimulated insulin secretion in HIT-T15 cells (**A**) and primary cultured islets (**B**). *$p < 0.05$, **$p < 0.01$, and ***$p < 0.001$ compared to 5 mM glucose alone (**A**) or 11.1 mM glucose alone (**B**). GPZ, glipizide. Effect of 0.5 mM diazoxide (**C**) or 10 μM nifedipine (**D**) on CK-induced insulin release from HIT-T15 cells. *$p < 0.05$ and **$p < 0.01$ compared to 5 mM glucose alone. ***$p < 0.001$ compared to 5 mM glucose with 8 μM CK. Data are mean $\pm$ standard error.

Measurement of Hepatic Enzyme Activities. Enzyme activities of glucose-6-phosphatase (G6Pase), glucokinase (GK) and glucose-6-phosphate dehydrogenase (G6PD) were measured according to the literature with slight modifications (*14–16*).

Assessment of Liver and Adipose Tissue Molecular Profiles. *RNA Extraction, Probe Preparation, and Hybridization.* RNA was isolated from the liver or white adipose tissue using an Easy-Blue Total RNA extraction kit (Intron Biotechnology Inc., Seoul, Korea). RNA quality and quantity were determined using the RNA 6000 Nano LabChip kit (Agilent Technologies, Palo Alto, CA). Fluorescent-labeled cRNA probe was prepared and hybridized from total RNA using amino allyl coupling reactions (AmershamPharmacia, Uppsala, Sweden). DNA chips were then scanned using the GenePix 4000B (Axon Instruments, Union City, USA).

Microarray Data Processing and Analysis. Scanned images were analyzed with Genepix Pro 3.0 software (Axon Instruments Inc., Union City, USA) to obtain gene expression ratios. Transformed data were then normalized using the Lowess procedure (*17*).

Real-Time PCR. Reverse transcription and real-time PCR reactions were performed using 1 μg of total RNA, LightCycler FastStart DNA Master SYBR Green I kit (BMSKorea, Seoul, Korea) in the Light Cycler real-time PCR detection system (Roche, Mannheim, Germany). Primer sets were validated, Cp values were normalized to housekeeping genes, β-actin, and fold-change ratios of experimental to control were calculated using the $\Delta\Delta$Cp method (*18*).

Statistical Analysis. Data were analyzed using Sigma Plot (Ver. 8.0, SPSS Inc., Chicago, IL). All data are expressed as mean $\pm$ standard error 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

I. In Vitro Studies and Single Administration of CK. *Effect of CK on Insulin Secretion.* To explore whether CK augments a glucose-stimulated insulin secretion, different concentrations of CK were treated to either HIT-T15 cells or primary cultured islets. In both cells, CK at the concentration range between 1 and 8 μM augmented a glucose-stimulated insulin secretion in a concentration-dependent manner with the maximal response occurring at 8 μM (**Figure 2A** and **Figure 2B**). CK (16 μM) stimulated the insulin secretion ($p < 0.05$), but magnitude was smaller than that in 8 μM of CK in primary cultured islets. Next, to examine how CK enhances a glucose-stimulated insulin secretion, diazoxide (K^+ channel opener) and nifedipine (L-type of Ca^{2+} channel blocker) were used. Diazoxide (0.5 mM) blocked a glucose-induced insulin secretion from 31.9 $\pm$ 2.7 to 15.4 $\pm$ 1.2 μU/mL in HIT-T15 cells ($p < 0.01$, **Figure 2C**). In HIT-T15 cells supplemented with 5 mM glucose and 8 μM CK, diazoxide suppressed the insulin secretion to a level observed in 5 mM glucose with diazoxide ($p < 0.001$). The addition of 10 μM nifedipine also reduced the insulin secretory effect of CK from 47.5 $\pm$ 2.5 to 19.3 $\pm$ 0.6 μU/mL ($p < 0.001$, **Figure 2D**), to a level observed in cells incubated with 5 mM glucose and nifedipine (18.5 $\pm$ 2.0 μU/mL).

Oral Glucose Tolerance Test (OGTT). OGTT was performed to determine the effect of a single oral dose of CK on glucose tolerance and insulin secretion using the ICR mice (**Figure 3**). Glucose challenge dramatically increased the blood glucose levels in control group mice, whereas CK-treated groups significantly suppressed the blood glucose levels from rising, especially at 30 min after glucose load ($p < 0.05$, **Figure 3A**). When the area under the curve (AUC) was compared between groups, CK12.5- and CK25-treated groups (12.5 and 25 mg/kg dose) showed 9% and 15% ($p < 0.05$) reduction, respectively, compared to that of control group (**Figure 3B**). Plasma insulin

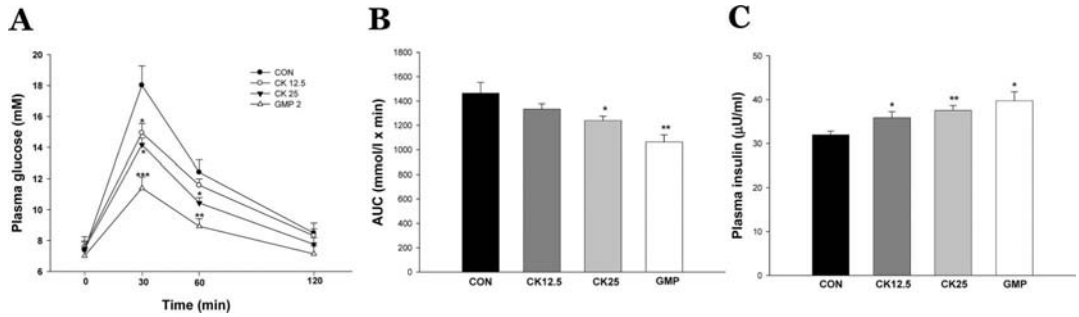

Figure 3. Plasma glucose responses to an oral glucose challenge (1.5 g/kg) after 12 h of food deprivation in ICR mice (**A**), the area under the curve (**B**) and plasma insulin levels at 30 min after glucose administration (**C**). Data are mean ± standard error ($n = 8$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control (CON) group. GMP, 2 mg/kg of glimepiride.

Table 1. Metabolic Parameters in CK-Treated *db*/*db* Mice[a]

parameter	CON	CK10	CK20	MT300
body weight (g)	42.3 ± 0.3	41.7 ± 0.6	42.3 ± 0.8	40.7 ± 0.8*
food intake (g)	969.1	969.0	893.4	907.5
water intake (mL)	1709	1285	1072	50054
plasma glucose (mM)	26.7 ± 1.7	19.9 ± 2.5*	16.4 ± 1.5***	11.9 ± 1.5***
plasma insulin (μU/mL)	222.4 ± 61.5	302.8 ± 70.7	224.6 ± 20.9	164.1 ± 34.5
HOMA-IR	263.5 ± 16.3	267.3 ± 33.8	164.0 ± 15.2**	86.6 ± 10.8***
HbA1c (%)	6.1 ± 0.1	5.3 ± 0.4*	5.1 ± 0.2**	5.0 ± 0.1***
plasma adiponectin (μg/mL)	64.9 ± 5.5	70.9 ± 2.9	85.9 ± 0.3**	71.2 ± 1.5
plasma lipids				
TG (mg/mL)	126.7 ± 13.5	5005.2 ± 8.8	121.7 ± 5.3	101.2 ± 5.7
NEFA (μequiv/L)	1639.0 ± 114.1	1269.4 ± 89.0*	1283.8 ± 94.3*	1664.7 ± 36.2
cholesterol (mg/mL)	154.6 ± 2.8	158.0 ± 5.4	152.6 ± 4.8	131.1 ± 2.6***
HDL-C (mg/mL)	96.4 ± 2.9	107.4 ± 3.9*	110.4 ± 4.3*	81.7 ± 2.7

[a] Data are mean ± standard error($n = 6$). Homeostasis model assessment 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 diabetic control (CON) group.

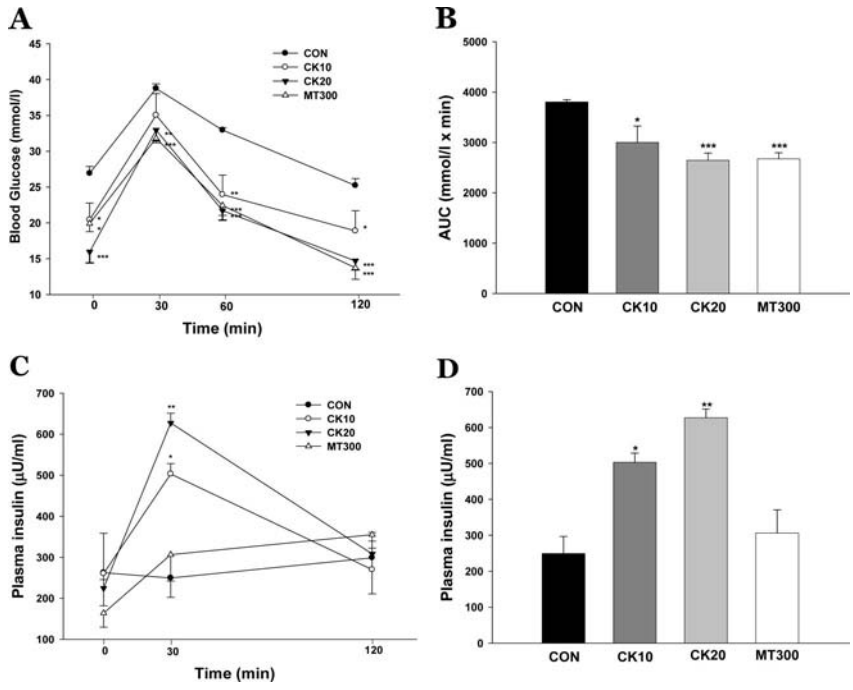

Figure 4. Plasma glucose and insulin responses to an oral glucose challenge (1.5 g/kg) after 12 h of food deprivation in *db*/*db* mice: **A**, plasma glucose responses to an oral glucose challenge; **B**, the area under the curve (AUC) of plasma glucose levels; **C**, plasma insulin responses to an oral glucose challenge; **D**, plasma insulin levels at 30 min after glucose administration. Data are mean ± standard error ($n = 6$). *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$ compared to control (CON) group.

level at 30 min after glucose load in the control group was 31.9 ± 0.8 μU/mL, whereas insulin levels in CK12.5- and CK25-treated groups were 35.8 ± 1.8 ($p < 0.05$) and 37.5 ± 1.1 μU/mL ($p < 0.01$), respectively, indicating that CK lowered the blood glucose levels by enhancing insulin secretion (**Figure 3C**).

II. Multiple Administration of CK. *Metabolic Parameter Changes.* Metabolic parameters related to diabetes are shown in **Table 1**. There was no significant difference in body weight between CON and CK-treated groups despite reduction of total food intake in the CK20 group. The MT300 group showed a

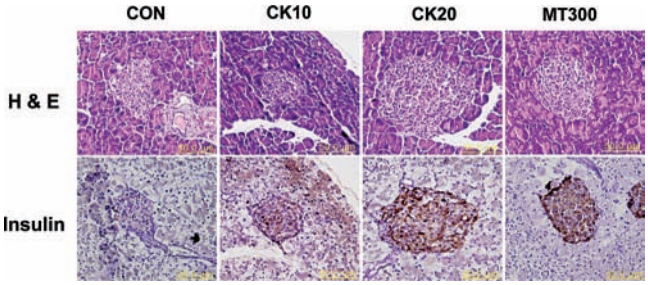

Figure 5. Morphology and insulin immunostaining of pancreatic β-cell in *db*/*db* mice. H & E, hematoxylin and eosin staining; insulin, anti-insulin antibody staining; Magnification $\times$200.

marginal reduction of body weight ($p < 0.05$), compared to the CON group concomitant with a decrease in total food intake. Total water intake in the CON group during the 25 day period was 1709 mL, but those in the CK-treated group were decreased in a concentration-dependent manner (CK10, 1285 mL; CK20, 1072 mL). Fasting plasma glucose levels in the CK-treated groups showed a significant decrease by 42% ($p < 0.05$) and 61% ($p < 0.001$), when compared to the CON group. However, there were no significant differences in the plasma insulin levels between groups. With decreased plasma glucose levels, the insulin resistance index (HOMA-IR) (*19*) of the CK20-treated group was significantly reduced by 38% ($p < 0.05$), when compared to the CON group. CK-treated groups also decreased the HbA1c levels by 0.8% in the CK10 group ($p < 0.05$) and 1.0% in the CK20 group ($p < 0.01$) compared to that of the CON group. On the other hand, plasma adiponectin level of the CK20-treated mice was significantly increased by 32% compared to the CON mice ($p < 0.01$). In addition, plasma lipid levels of the CK20-treated mice were also significantly improved by 22% reduction in NEFA and 15% increase in HDL-C, compared to the CON mice ($p < 0.05$). Plasma TG and total cholesterol, however, were not significantly decreased.

OGTT. To determine the effect of multiple oral administration of CK on glucose tolerance, OGTT was carried out at the end of the experiment (**Figure 4**). Glucose challenge dramatically increased the blood glucose levels in the CON group, whereas CK-treated groups significantly suppressed the blood glucose levels from rising during 120 min after glucose challenge (**Figure 4A**). When the AUC was compared between groups, CK10- and CK20-treated groups showed 21% and 30% reduction, respectively, compared to that in the CON group ($p < 0.05$ and $p < 0.001$, **Figure 4B**). Improvement of the glucose intolerance in the CK20-treated group was quite similar to that in the MT300-treated group (30% reduction compared to the CON group, $p < 0.001$). Plasma insulin levels in the CK-treated groups was not decreased, but rather significantly increased at 30 min time point (**Figure 4C**). Plasma insulin levels at 30 min after glucose challenge in the CK10- and CK20-treated groups were markedly increased by 2- and 2.5-fold, respectively, compared to the CON group ($p < 0.05$ and $p < 0.01$, **Figure 4D**).

Morphology of Pancreas and Anti-Insulin Immunostaining. The islets of the CON group mice revealed infiltrated mononuclear cells, indicating β-cell damage, and insulin content in these islets of CON mice was barely detected (**Figure 5**). However, CK treatment prevented the destruction of islets and preserved more insulin in a concentration-dependent manner.

Hepatic Gene Expressions and Enzyme Activities. Among 19858 genes, we selected 18 hepatic genes functionally significant in diabetes. As shown in **Table 2**, CK treatment changed gene expressions associated with the glycolysis/gluconeogenesis and pentose phosphate pathway, including decreased expressions of G6Pase (0.76-fold) and fructose bisphosphatase 1 (0.68-fold), and increased expressions of GK (1.30-fold) and G6PD (1.26-fold). To confirm the microarray data, enzyme activities of G6Pase, GK, and G6PD were also determined in the liver of *db*/*db* mice (**Figure 6**). Both G6Pase and GK activities were significantly changed in the CK20-treated mice, compared to the CON mice. The G6Pase activity was decreased by 54% ($p < 0.05$, **Figure 6B**), whereas the GK activity was increased by 36% ($p < 0.01$, **Figure 6A**) in the CK20-treated group compared to the CON group. The G6PD activity was also increased by 45% in the CK20-treated group compared to the CON group, although the increment was not significant ($p = 0.054$, **Figure 6**C).

Adipocyte Gene Expressions and Real-Time PCR. We compared the gene expression profiles in the adipose tissue between CK20-treated and vehicle-treated *db*/*db* mice using microarray. Among 19858 genes, 19 genes functionally related to diabetes were selected (**Table 3**). CK treatment increased expression of genes responsible for adipocytokine signaling

Table 2. Differentially Expressed Genes in the Liver of CK-Treated *db*/*db* Mice

accession no.	gene name	fold change	function
	Up-Regulated		
NM_011044	phosphoenolpyruvate carboxykinase 1, cytosolic	1.39	gluconeogenesis
NM_013631	pyruvate kinase liver and red blood cell	1.27	glycolysis
NM_010292	glucokinase	1.3	glycolysis
NM_008826	phosphofructokinase, liver, B-type	1.34	glycolysis
NM_013820	hexokinase 2	1.34	glycolysis
NM_144903	aldolase 2, B isoform	2.05	glycolysis
NM_008155	glucose phosphate isomerase 1	1.26	glycolysis/gluconeogenesis
NM_173371	hexose-6-phosphate dehydrogenase (glucose 1-dehydrogenase)	1.23	pentose phosphate pathway
NM_025396	6-phosphogluconolactonase	1.25	pentose phosphate pathway
NM_008062	glucose-6-phosphate dehydrogenase X-linked	1.26	pentose phosphate pathway
BC011329	phosphogluconate dehydrogenase	1.42	pentose phosphate pathway
	Down-Regulated		
NM_019395	fructose bisphosphatase 1	0.68	gluconeogenesis
NM_008061	glucose-6-phosphatase, catalytic	0.76	gluconeogenesis
NM_007994	fructose bisphosphatase 2	0.81	gluconeogenesis
NM_029344	acylphosphatase 2, muscle type	0.73	glycolysis/gluconeogenesis
NM_031190	phosphoglycerate kinase 2	0.8	glycolysis/gluconeogenesis
NM_145572	glycogen synthase 2	0.77	insulin signaling pathway
NM_026662	phosphoribosyl pyrophosphate synthetase 2	0.68	pentose phosphate pathway

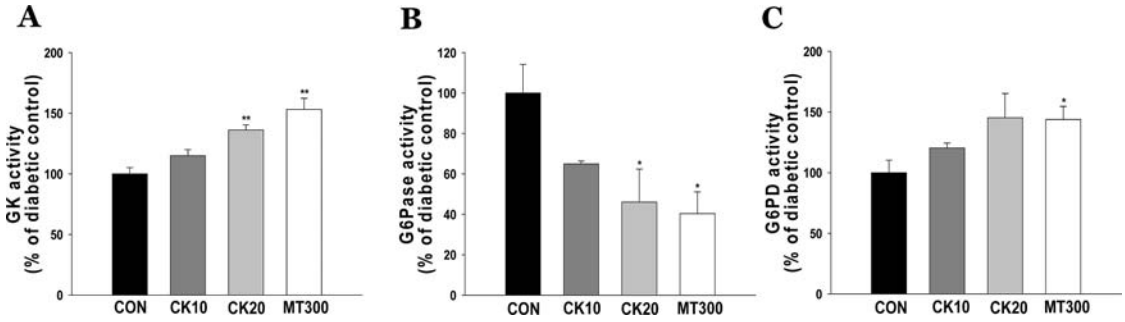

Figure 6. Effect of CK on GK (**A**), G6Pase (**B**), and G6PD (**C**) activities in *db*/*db* mice. Data are mean ± standard error ($n = 3$). *$p < 0.05$ and **$p < 0.01$ compared to control (CON) group.

Table 3. Differentially Expressed Genes in the Adipose Tissue of CK-Treated *db*/*db* Mice

accession no.	gene name	fold change: micro array	fold change: real time PCR	function
	Up-Regulated			
NM_011146	peroxisome proliferator activated receptor gamma	1.27	1.35	adipocytokine signaling
NM_011492	serine/threonine kinase 11	1.34		adipocytokine signaling
NM_009204	solute carrier family 2 (facilitated glucose transporter), member 4	1.94	1.29	adipocytokine signaling
AK036585	protein kinase, AMP-activated, gamma 3 noncatatlytic subunit	1.56		adipocytokine signaling
L23108	CD36 antigen	1.37	0.26	adipocytokine signaling
AK050846	calcium/calmodulin-dependent protein kinase kinase 2, beta	1.42		adipocytokine signaling
NM_009605	adiponectin, C1Q and collagen domain containing	1.57		adipocytokine signaling
NM_178143	protein kinase, AMP-activated, alpha 2 catalytic subunit	1.23		adipocytokine signaling
NM_133360	acetyl-coenzyme A carboxylase alpha	1.42		fatty acid biosynthesis
BC022940	acetyl-coenzyme A carboxylase beta	1.62		fatty acid biosynthesis
NM_007988	fatty acid synthase	2.26	5.06	fatty acid biosynthesis
NM_009127	stearoyl-coenzyme A desaturase 1	1.21	3.23	fatty acid biosynthesis
NM_053115	acyl-coenzyme A oxidase 2, branched chain	1.59	1.42	fatty acid metabolism
NM_009949	carnitine palmitoyltransferase 2	1.31		fatty acid metabolism
NM_007383	acyl-coenzyme A dehydrogenase, short chain	1.53		fatty acid metabolism
	Down-Regulated			
NM_018883	calcium/calmodulin-dependent protein kinase kinase 1, alpha	0.83		adipocytokine signaling
NM_019477	acyl-CoA synthetase long-chain family member 4	0.81		fatty acid metabolism
NM_144823	acyl-CoA synthetase long-chain family member 6	0.82		fatty acid metabolism
NM_028817	acyl-CoA synthetase long-chain family member 3	0.73		fatty acid metabolism

(PPAR-γ, 1.27-fold; GLUT4, 1.94-fold; CD36, 1.37-fold) and fatty acid synthesis/metabolism pathways (FAS, 2.26-fold; SCD-1, 1.21-fold; ACOx2, 1.59-fold). To confirm these data, quantitative real-time PCR was performed and the expression pattern of these genes was quite similar with those of the array data except for CD36.

DISCUSSION

In our preliminary study on screening ginsenosides with insulin secretory effect, CK showed the most potent insulin secretory activity in HIT-T15 cells (data not shown). On the basis of our previous results, we decided to explore how CK enhanced an insulin secretion in HIT-T15 cells and primary cultured islets. Our results demonstrated that CK treatment caused a concentration and glucose-dependent stimulation of insulin secretion presumably through K_{ATP}-channel-dependent pathway (**Figure 2**). The abolishment of CK-induced insulin secretion by diazoxide provides the rationale on this view, which was further verified by the experiment using a Ca^{2+} channel blocker, nifedipine (**Figure 2D**). Insulin secretory activity of CK was also confirmed by performing OGTT in ICR mice. CK suppressed the plasma glucose levels from rising after glucose loads at doses of 12.5 and 25 mg/kg (**Figure 3A**), and such a glucose lowering effect of CK was due to the increment of insulin secretion (**Figure 3C**).

Sulfonylureas are well-known insulin secretagogues of whose actions are mainly on the pancreas. However, there have been several reports about extrapancreatic actions of these drugs in long-term treatment (*20, 21*). They claimed that sulfonylureas improve insulin sensitivity with actions on muscle and liver, accounting for enhanced peripheral glucose disposal. On the basis of these reports, we tried to investigate whether CK has additional effects on diabetes using an obese diabetic model, C57BL/KsJ *db*/*db* mice.

The increased glucose levels together with the elevated NEFA levels can synergize to further adversely affect β-cell health and insulin action, often referred to "glucolipotoxicity". In diabetes, elevated glucose and NEFA levels can contribute to the generation of reactive oxygen species (ROS). Since pancreatic islets are highly susceptible to oxidative stress, ROS may be involved in progressive β-cell dysfunction or loss in T2DM. **Table 1** shows that CK treatment caused the reduction of plasma glucose and NEFA levels compared to those in diabetic control mice, and because of reduced glucolipotoxicity, CK-treated mice showed less destructed β-cell and more insulin content (**Figure 5**). The result of oral glucose tolerance test is relevant to this interpretation. As shown in **Figure 4**, CK improved the glucose tolerance in a dose-dependent fashion and this effect was probably due to a marked increment of insulin secretion at 30 min after glucose challenge. In order for glucose tolerance to remain unchanged, changes in insulin sensitivity must be matched by a proportionate yet opposite change in circulating

insulin levels. Failure of this feedback loop results in a deviation from normal glucose tolerance and underlies the development of diabetes.

Next, to explore the extrapancreatic effect of CK, microarray analysis in the liver was conducted. GK, phosphoenolpyruvate carboxylase (PEPCK), G6pase, and G6PD are the key enzymes of glucose metabolism in the liver. GK, also termed hexokinase, induces glucose utilization both by triggering glucose storage into glycogen and by increasing glycolysis (*22*). PEPCK and G6pase catalyze rate-limiting and final step of gluconeogenesis, respectively (*23*), and G6PD is a hepatic enzyme catalyzing the first step of pentose phosphate pathway, which is another possible fate for glucose metabolism (*24*). In diabetic patients, the rate of hepatic gluconeogenesis is considerably increased with elevated G6pase and PEPCK gene expressions, whereas GK and G6PD gene expressions are reduced (*24–26*). **Table 2** shows differential expression of genes associated with diabetes in CK-treated over control mice. Genes responsible for glycolysis and pentose phosphate pathway were up-regulated, and genes for gluconeogenesis were down-regulated. To verify these array data, hepatic enzyme activities for GK, G6Pase, and G6PD were determined. As shown in **Figure 6**, GK and G6PD activities were activated, but G6Pase activity was reduced in a dose-dependent manner, resulting in a shift from hepatic glucose production to hepatic glucose utilization.

Microarray analysis in the adipose tissue was also conducted, and results are shown in **Table 3**. The past decade has seen adipose tissue move from being a passive participant to having a pivotal role in diverse homeostasis processes, with particular emphasis on energy balance and glucose homeostasis. We now understand that the profound effect of adipocytes on glucose homeostasis is mediated by several different mechanisms, which can be roughly categorized as endocrine and nonendocrine (*27*). The adipose tissue as an important endocrine organ secretes a number of biologically active "adipokines". Of these adipokines, adiponectin has recently attracted much attention because of its antidiabetic and antiatherogenic effects. Plasma adiponectin levels have been reported to be reduced in obese humans, particularly those with visceral obesity, and to correlate inversely with insulin resistance (*28*). Therefore, it is worth noting that adiponectin gene expression was increased by 1.57-fold over the control in the array data (**Table 3**) concomitantly increased plasma adiponectin levels (**Table 1**). Adiponectin promotes adipocyte differentiation, insulin sensitivity, and adipogenesis (*29*). It has a thread of connection with our data, up-regulations of adipogenic genes such as peroxisome proliferator activated receptor-gamma (PPAR-γ), fatty acid synthase (FAS), and stearoyl-coenzyme A desaturase 1 (SCD-1) (**Table 3**). From these results, we can speculate that increased levels of adiponectin by CK induced adipogenesis, increasing ability to store lipids and to sensitize the insulin action in the adipose tissue. Improved plasma lipid profiles, especially in NEFA (**Table 1**), and decreased hepatic triglyceride content (data not shown) probably result from increasing NEFA uptake into adipose tissue. It has also been reported that adiponectin increases GLUT4 expression, which is the primary glucose transporter expressed in the skeletal muscle and adipose tissue (*29, 30*). In our array data, CK up-regulated GLUT4 gene expression in the adipose tissue (1.94-fold, **Table 3**). Real-time PCR for PPAR-γ, GLUT4, CD36, FAS, SCD-1, and ACOx were performed and normalized real-time PCR data showed the same pattern as the array. These results are consistent with increased adiponectin levels, and CK is expecting for the treatment of diabetes.

In summary, CK enhanced the insulin secretion in pancreatic β-cell through an action on K_{ATP}-channel-dependent pathway and this result was conformed in OGTT. In *db*/*db* mice, multiple administration of CK showed the hypoglycemic activity and improved glucose tolerance with β-cell preservation. In addition, CK increased glucose utilization in the liver and improved insulin sensitivity through lipid storage and activation of glucose transporter in the adipose tissue. In view of our present data, we may suggest that CK could provide a solid basis for the development of new antidiabetic drug.

ACKNOWLEDGMENT

This work was supported by a grant (M106KD010018-07K0401-01810) from the Plant Diversity Research Center of the 21st Century Frontier Program. Gi Cheol Han is supported by the BK21 Research Fellowship from the Ministry of Education and Human Resources Development.

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Received for review July 27, 2007. Revised manuscript received October 19, 2007.

JF0722598