

Effect and Mechanism of Ginsenosides CK and Rg1 on Stimulation of Glucose Uptake in 3T3-L1 Adipocytes

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The glucoregulatory activities of ginsenosides compound K (CK) and Rg1 were investigated in 3T3-L1 adipocytes. Both compounds significantly enhanced glucose uptake in 3T3-L1 adipocytes in a dose–response manner, which is correlated with increased GLUT4 translocation from intracellular vesicles to the plasma membrane in adipocytes. The stimulating effects of CK and Rg1 on glucose uptake and GLUT4 translocation are associated with activation of AMP-activated protein kinase (AMPK) and phosphatidylinositol 3-kinase (PI3K) signaling pathways; both are key pathways in mediating glucose uptake in animal cells. In addition to the acute stimulus effect of glucose uptake, prolonged incubation of CK and Rg1 significantly induced GLUT4, but not GLUT1, expression at both the mRNA and protein levels in 3T3-L1 adipocytes. Further studies showed that CK inhibited and Rg1 enhanced triglyceride accumulation in adipocytes, suggesting that the mechanism of action of these ginsenosides may not be completely identical. In summary, this study demonstrated insulin-like activities of CK and Rg1 in adipocytes. These findings are important in understanding the hypoglycemic properties and potential applications of ginseng and ginsenosides.

KEYWORDS: Ginsenoside; compound K; Rg1; GLUT4; glucose uptake; 3T3-L1 adipocytes

INTRODUCTION

Insulin plays a key regulatory role in stimulating the transport of blood glucose into peripheral tissues through the GLUT4 transporter, which is mainly expressed in skeletal muscle and adipose cells. In these target cells, insulin stimulates the translocation and redistribution of the GLUT4 glucose transporter from specific intracellular compartments to the plasma membrane, where it facilitates glucose uptake (1). The insulin-mediated glucose uptake is impaired in insulin resistance, thus resulting in decreased glucose uptake into muscle or adipose cells. Insulin resistance is a major metabolic disorder of diabetes, which is associated with serious conditions such as cardiovascular and kidney disease. Despite effective therapeutic drugs for diabetes treatment, there are undesirable side effects and their precise action mechanism remains to be completely clarified. Natural products provide an important source for searching new drugs to prevent metabolic disorders and as new therapeutic approaches (2).

Ginseng is one of the most widely used herbal medicines with a long history in Asian countries. Ginseng exerts a wide range of beneficial effects such as antiaging, improving cognitive performance, and enhancing metabolic functions (3). The main active components in ginseng are ginsenosides, which are generally believed to contribute to ginseng's pharmacological actions (3). Historically, used in treating diabetes, ginseng is known for its

prominent hypoglycemic activity (4). Ginseng was shown to decrease postprandial glycemia in both nondiabetic and type 2 diabetes subjects (5). The aqueous extract of ginseng was shown to be capable of producing hypoglycemia in both glucose-loaded healthy animals and in animals with experimentally induced diabetes (6). The proposed mechanisms for the hypoglycemic effect of ginseng may involve enhanced insulin secretion (7), increased insulin sensitivity (6), and reduced glucose absorption (8) or a combination of these modes. Despite extensive literature documenting the glucoregulatory activity of ginseng, the mechanisms responsible for ginseng's hypoglycemic effect still need to be elucidated.

Previously, we demonstrated that these ginsenosides display a significant but opposite effect on the rate of glucose transport across the Caco-2 cell. We found that the protopanaxidiol type compound K (CK) enhanced but the protopanaxitriol type Rg1 suppressed glucose uptake in Caco-2 cells (9). To get deeper and further understanding of the action mechanism of the hypoglycemic activity of ginseng, the effect and mechanism of the ginsenosides CK and Rg1 on glucose uptake and the intracellular redistribution of GLUT4 in 3T3-L1 adipocytes were investigated. Our results demonstrated that CK and Rg1 displayed significant stimulatory effect on GLUT4 membrane redistribution, which in turn increased glucose uptake activity in 3T3-L1 adipocytes. Further studies demonstrated the involvement of phosphatidylinositol-3 kinase (PI3K) and AMP-activated protein kinase (AMPK) in CK- or Rg1-mediated activities. In addition, the effects of CK and Rg1 on GLUT1 and GLUT4 gene expression and on triglyceride accumulation in 3T3-L1 adipocytes were also investigated. Our findings provide the molecular basis for hypoglycemic

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activity of ginsenosides CK and Rg1, which are promising candidates in the development of an antidiabetic drug.

MATERIALS AND METHODS

Extraction and Isolation of Ginsenosides. Dried roots of *Panax notoginseng* (5.05 kg) were extracted repeatedly with EtOH (10 L \times 5) at room temperature. The combined EtOH extract was evaporated in vacuo to yield a dark brown syrup (778.19 g). The syrup was partitioned between *n*-hexane and 80% MeOH to give an *n*-hexane layer (20.01 g), a MeOH layer (741.41 g), and an insoluble layer (16.77 g). The MeOH layer was chromatographed on an Amberlite XAD-4 column by elution with H₂O, 50% MeOH/H₂O, and 100% MeOH, gradually, to give three fractions: H₂O fraction (202.02 g; fraction 1), 50% MeOH/H₂O fraction (68.35 g; fraction 2), and 100% MeOH fraction (471.04 g; fraction 3). Fraction 2 was chromatographed on Lobar (RP-18, 75% MeOH/H₂O) to give ginsenoside Rb1 (5.53 g). Fraction 3 was subjected to SiO₂ column chromatography and eluted with CHCl₃/MeOH/H₂O (65:35:10) and MPLC (C-8, 75% MeOH/H₂O) to give ginsenoside Rg1 (29.54 g) and ginsenoside Rb1 (25.14 g). CK was produced by enzymatic hydrolysis of ginsenoside Rb1 with naringinase as described (9).

Cell Culture. 3T3-L1 preadipocytes were obtained from American Type Culture Collection (ATCC, Manassas, VA) and cultured in Dulbecco's minimal essential medium (DMEM) supplemented with 100 IU/mL penicillin, 100 μ g/mL streptomycin, 1% nonessential amino acid, and 10% calf serum (CS, Hyclone, Logan, UT) in 5% CO₂ at 37 °C. Differentiation was induced by treating the cells with differentiation inducers (DMEM containing 0.5 mM 3-isobutyl-1-methylxanthane (IBMX), 0.25 μ M dexamethasone, 10 μ g/mL insulin, and 10% fetal bovine serum (FBS, Gibco, Gaithersburg, MD)) for 48 h. The cells were refed with DMEM supplemented with 10 μ g/mL insulin and 10% FBS for the following 48 h and changed every 2 days. More than 90% of the cells expressed the adipocyte phenotype between 8 and 10 days after the initiation of differentiation and were used for the experiments. 3T3-L1 cells stably expressing HA-GLUT4-GFP were transfected by Lipofectamine 2000 (Invitrogen, Carlsbad, CA) and selected by G418 treatment. The plasmid to express HA-GLUT4-GFP, an exofacial HA epitope and a C-terminal GFP, was a generous gift from Dr. Timothy E. McGraw of Weill Cornell Medical College, New York (10).

2-Deoxyglucose Uptake Assay. Glucose uptake in 3T3-L1 adipocytes was performed using [¹⁴C] 2-deoxy-D-glucose (2-DG), a nonmetabolizable analogue of D-glucose, as the substrate. Cells cultured in 24-well plates and differentiated for 8–10 days were incubated in the serum-free DMEM containing 0.2% BSA, followed by incubation in the media without serum but with compound for various doses. In inhibitor study, adipocytes were preincubated in the presence or absence of inhibitors (1 μ M LY294002 or compound C) for 1 h and then incubated with 0.1 μ M CK or Rg1 for 2 h. Cells were incubated for 3 h at 37 °C in transport buffer Krebs–Ringer's phosphate (KRP) buffer (20 mM HEPES, 137 mM NaCl, 4.7 mM KCl, 1.2 mM MgSO₄, 1.2 mM KH₂PO₄, 2.5 mM CaCl₂, and 2 mM pyruvate, pH 7.4, and 0.2% BSA) before the addition of 100 nM insulin. Transport experiments was started by replacing the KRP buffer containing 0.1 mM 2-DG in which 0.2 μ Ci of [¹⁴C] 2-DG (200 μ Ci/mL, Amersham Biosciences, Little Chalfont, Bucks, U.K.) was added for 5 min. Cells were washed twice with ice-cold PBS and solubilized with 0.2% SDS, and the amount of radioactivity was measured with a scintillation counter (TopCount, Packard BioSciences, Meriden, CT). Protein concentration of each sample was quantified using a BCA (bicinchoninic acid) protein assay kit (Pierce, Rockford, IL).

Western Blot Analysis. 3T3-L1 preadipocytes were seeded on 6-well plates and left to undergo differentiation for 8–10 days prior to treatment with various doses of ginsenosides for 2 or 24 h. To block protein synthesis, the cells were pretreated for 1 h with cycloheximide (CHX, 5 μ g/mL) before ginsenosides were added. The cells were then washed with ice-cold PBS and lysed in 0.2 mL of lysis buffer (1% NP-40, 150 mM NaCl, 0.1% SDS, 50 mM Tris-HCl, pH 7.6, 10 mM EDTA, 0.5% deoxycholate, 1 mM PMSF, 1 mM Na₃VO₄, 10 mM NaF, 10 mM β -glycerophosphate, 10 μ g/mL protease inhibitor, and phosphatase inhibitor cocktails) for 30 min at 4 °C. Equal amounts of protein of each sample were separated by sodium dodecyl sulfate (SDS)–10% polyacrylamide gel electrophoresis (PAGE) and transferred onto polyvinylidene difluoride (PVDF) membranes (Millipore, Bedford, MA). Immunoblotting was performed with antibodies for

GLUT1 (Abcam, Cambridge, MA), GLUT4 (R&D systems, Minneapolis, MN), clathrin (Santa Cruz, CA), p-AKT (S473), AKT, p-AMPK (T172), AMPK (Cell Signaling Tech, Danvers, MA), and β -actin (Chemicon, Temecula, CA). Signals were visualized with an enhanced chemiluminescence kit (ECL, Amersham, U.K.) followed by exposure to X-ray films.

Subcellular Fractionation of Membrane Systems. Subcellular fractionation of plasma membrane (PM) and low-density microsomal membrane (LDM) was performed essentially as described (11). Differentiated 3T3-L1 adipocytes were treated with insulin (100 nM) or with various doses of ginsenoside for 2 h alone or in combination with 1 h pretreatment of CHX (5 μ g/mL) or 1 μ M of specific kinase inhibitors. The cells were washed in ice-cold PBS and scraped in HES buffer (20 mM Tris-HCl, pH 7.4, 255 mM sucrose, 1 mM EDTA, and protease inhibitor mixture) on ice and homogenized. The homogenate was centrifuged at 16000g for 20 min. The PM fraction was collected from the interface of a 1.12 M sucrose cushion following centrifugation of the 16000g pellet at 70000g for 10 min. PM was then resuspended in homogenization buffer and pelleted at 200000g for 4 min. The obtained fractions were then resuspended in HES buffer to a mixture of about 1–2 mg/mL protein and subjected to Western blotting analysis.

Quantitative Analysis of Glucose Transporter Transcripts. Relative levels of GLUT1 and GLUT4 transcripts expressed in the 3T3-L1 adipocytes were performed by quantitative real time PCR (Q-PCR). After the cells were treated with various doses of appropriate agents, total RNA was isolated using Trizol (Invitrogen, Irvine, CA). RNA (1 μ g) was reverse-transcribed to cDNA using High-Capacity cDNA Reverse Transcription Kits (Applied Biosystems, Darmstadt, Germany). The SYBR Green Q-PCR assay was performed on the StepOne real-time PCR System with SYBR green PCR master mix (Applied Biosystems, Foster City, CA). Relative gene expression values were determined by the $\Delta\Delta C_T$ method using the StepOne v2.0 software (Applied Biosystems). Primers were designed using Primer Express 2.0 software (Applied Biosystems). The following primers were used for amplification of mouse GLUT4 gene (NM_009204): 5'-CAGCCTACGCCACCATAG-3' (forward, F) and 5'-TTCCAGCAGCAGCAGAGC-3' (reverse, R). Those used for mouse GLUT1 gene (NM_011400) were 5'-TGTGGTGTGCTGTTTGTGTTAG-3' (F) and 5'-CAATGAAGTTTGAGTCCAGTTGG-3' (R) and for mouse β -actin gene (NM_007393), 5'-ACCACACCTTCTCAATGAG-3' (F) and 5'-ACGACCAGAGGCATACAG-3' (R). Data are presented as the fold differences in gene expression normalized to the housekeeping gene β -actin as endogenous reference and relative to the untreated control cells.

Translocation Assay. Establishment of HA-GLUT4-GFP stable transfected 3T3-L1 preadipocytes and analysis of surface-exposed HA-GLUT4-GFP protein were performed as described with minor modifications (12). Briefly, the HA-GLUT4-GFP stable transfected 3T3-L1 preadipocytes were plated to 96-well plates and differentiated as described above. After treatment with appropriate agents, cells were fixed in 4% paraformaldehyde in PBS for 15 min at room temperature and then incubated for 2 h with primary anti-hemagglutinin (HA) antibody (12CA5) followed by incubation with rhodamine-conjugated secondary antibodies (Leinco, Ballwin, MO) for 1 h at room temperature. Primary and secondary antibodies were used at 1:100 dilutions in blocking solution. Fluorescence (Em 480/Ex 425 nm and Em 576 nm/Ex 550 nm) was measured using a fluorescence microtiter plate reader (POLARstar Galaxy; BMG Labtechnologies, Offenburg, Germany). The ratio of rhodamine fluorescence to GFP fluorescence was calculated. This ratio is a measure of surface-exposed HA-GLUT4-GFP normalized for the total GFP expressed.

Analysis of Triglyceride Contents and Glycerol Release. Triglyceride (TG) contents were analyzed by Oil-red O staining as described (13). After 48 h of compound treatment, cells were washed with ice-cold PBS and fixed with 3.7% formaldehyde in PBS and then incubated in Oil-red O solution (0.2% Oil-red O in 60% isopropanol). The stained lipid droplets were extracted in isopropanol and quantified spectrophotometrically at 490 nm. The amount of glycerol released into culture medium was measured using a glycerol assay kit (Randox Laboratories, Antrim, U.K.) following the manufacturer's direction.

Statistical Analysis. All results are representative of at least three independent experiments. Statistical analysis was performed using the SPSS 11.0 for Windows software. One-way analyses of variance (ANOVA) were carried out in compound treatment. Multiple comparisons of the

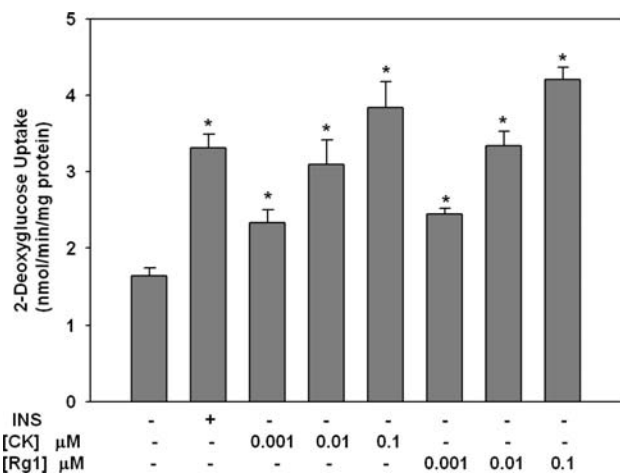


Figure 1. Effects of CK and Rg1 on 2-DG uptake in 3T3-L1 adipocytes. 3T3-L1 adipocytes were treated with indicated concentrations of CK and Rg1 or 100 nM insulin (INS) for 2 h and then analyzed for 2-DG uptake. Data represent mean \pm SE ($n = 3$). *, $p < 0.05$ versus control.

groups treated with different dosages were undertaken by Tukey tests. The comparison of an experimental group against the positive control was determined by a two-sample t test. Data were expressed as mean \pm SE, and a p value of < 0.05 was considered to be statistically significant.

RESULTS

CK and Rg1 Enhance Glucose Uptake in 3T3-L1 Adipocytes. Treatment of fully differentiated 3T3-L1 adipocytes for 2 h with increasing doses of CK and Rg1 resulted in significant enhancement in glucose uptake (**Figure 1**). CK increased glucose uptake in a dose-dependent manner from 142 to 233% of the control ($p < 0.05$, **Figure 1**) over the concentration range of 0.001–0.1 μM . Similarly, Rg1 displayed a gradual increase in glucose uptake from 0.001 to 0.1 μM (149–255% of the control, $p < 0.05$) in adipocytes. The results indicated that the submicromolar doses of CK and Rg1 have a remarkable effect on glucose uptake in 3T3-L1 adipocytes.

CK and Rg1 Enhance GLUT4 Translocation to Plasma Membrane in 3T3-L1 Adipocytes. To study the effect of CK and Rg1 on GLUT4 membrane translocation in adipocytes, plasma membrane (PM) was fractionated by ultracentrifugation and subjected to immunoblotting of GLUT4 protein. As shown in **Figure 2A,B**, CK and Rg1 caused a significant increase in GLUT4 redistribution in the PM fraction in a dose-dependent manner over the concentration range of 0.001–0.1 μM . The amount of GLUT4 protein in PM increased from 127 to 193% and from 127 to 224% of the control for CK and Rg1, respectively, $p < 0.05$. As an experimental control for the translocation of GLUT4 to PM, the GLUT4 level in the LDM fraction was also examined. Results in **Figure 2A** show that the GLUT4 level in LDM decrease gradually as the GLUT4 level in PM increased. These results demonstrated that both CK and Rg1 enhanced GLUT4 translocation from the intracellular pool to the PM.

Adipocytes that overexpress HA-GLUT4-GFP were also used to confirm the effects of CK and Rg1 on GLUT4 translocation. The increase of HA-GLUT4-GFP in the PM was measured by an increase in HA epitope in the PM. PM HA-GLUT4-GFP was measured by indirect immunofluorescence of the HA epitope in intact cells, and the total amount of HA-GLUT4-GFP per cell was determined by the GFP fluorescence. The anti-HA/GFP ratio is proportional to the fraction of GLUT4 in the PM. As shown in **Figure 2C**, CK and Rg1 induced a dose-dependent increase in the anti-HA/GFP ratio, with maximum increases of

2.1- and 1.8-fold at 0.1 μM for CK and Rg1, respectively. These results indicated that CK and Rg1 induced GLUT4 translocation to PM in 3T3-L1 adipocytes.

To examine whether the change of GLUT4 protein level in PM fraction in the CK- and Rg1-treated cells is dependent on de novo protein synthesis, experiments were performed in the presence of CHX to block de novo protein synthesis in 3T3-L1 adipocytes. The results shown in **Figure 3A** indicate that the amount of GLUT4 in PM fraction was significantly increased (187 or 182% of control, respectively, $p < 0.05$, **Figure 3A**) in adipocytes treated with CK or Rg1 for 2 h in the presence of CHX; however, the total intracellular GLUT4 level did not change significantly (**Figure 3B**). In addition to the acute effect, **Figure 3C** shows the results of the prolonged treatment of CK and Rg1 in adipocytes in the presence of CHX. Total GLUT4 protein levels in adipocytes remained unchanged or increased slightly after 24 h of incubation with CK, Rg1, and insulin (125, 95, and 122% of control, respectively, $p < 0.05$, **Figure 3C**). These results indicate that the acute effect of CK and Rg1 on glucose uptake was due primarily to the enhanced GLUT4 translocation but not to de novo GLUT4 protein synthesis.

CK and Rg1 Induce GLUT4 Gene Expression in 3T3-L1 Adipocytes. The effects of prolonged incubation of these ginsenosides on GLUT1 and GLUT4 gene expression in adipocytes are also investigated. Results shown in **Figure 4** indicate that GLUT4 protein level was significantly increased in both of CK- and Rg1-treated 3T3-L1 adipocytes. After 24 h of incubation, CK significantly increased the GLUT4 protein level with maximum increment at 0.1 μM (168% of the control, $p < 0.05$, **Figure 4A,B**) over the concentration range studied. Similarly, Rg1 also enhanced GLUT4 protein level with maximum increment at 0.1 μM (180% of the control, $p < 0.05$, **Figure 4A,B**). In contrast to GLUT4 protein, CK and Rg1 showed little and insignificant effects on GLUT1 protein expression in 3T3-L1 adipocytes.

Q-PCR was used to analyze the relative level of GLUT4 mRNA expressed in 3T3-L1 adipocytes. Results shown in **Figure 4C** indicate that both CK and Rg1 treatments resulted in significantly increased GLUT4 mRNA levels at 0.1 μM (301 and 236% of the control, respectively, $p < 0.05$, **Figure 4C**). As expected, there is no significant change in GLUT1 mRNA in 3T3-L1 adipocytes treated with these compounds. These results indicate that, in addition to an acute effect on GLUT4 membrane translocation, CK and Rg1 also exert a long-term effect on induction of GLUT4 gene expression in 3T3-L1 adipocytes.

CK and Rg1 Display Differential Effects on Triglyceride Accumulation and Lipolysis in 3T3-L1 Adipocytes. To explore the fate of increased intracellular glucose in cells treated with CK and Rg1, 3T3-L1 adipocytes were incubated with CK or Rg1 for 48 h and the amounts of intracellular TG accumulated and glycerol released into medium were determined. From the results shown in **Figure 5A**, Rg1 significantly increased TG accumulation over the concentration range of 0.001–0.1 μM with the maximum increase at 0.001 μM (214 to 159% of the control, respectively, $p < 0.05$). Consistent with increased TG, 0.1 μM Rg1 inhibited glycerol release into the medium significantly (62% of the control, $p < 0.05$, **Figure 5B**). In contrast to Rg1, CK significantly decreased TG accumulation in 3T3-L1 adipocytes in a dose-dependent manner with maximum inhibition at 0.1 μM (69% of control, $p < 0.05$, **Figure 5A**) over the concentration range assayed. As expected, 0.1 μM CK significantly enhanced glycerol release into medium (154% of the control, $p < 0.05$, **Figure 5B**). These results indicate that the underlying mechanisms may not be identical for CK and Rg1 on adipogenesis and lipolysis in 3T3-L1 adipocytes.

PI3K and AMPK Pathways Are Involved in CK- and Rg1-Induced Glucose Uptake and GLUT4 Translocation. To elucidate

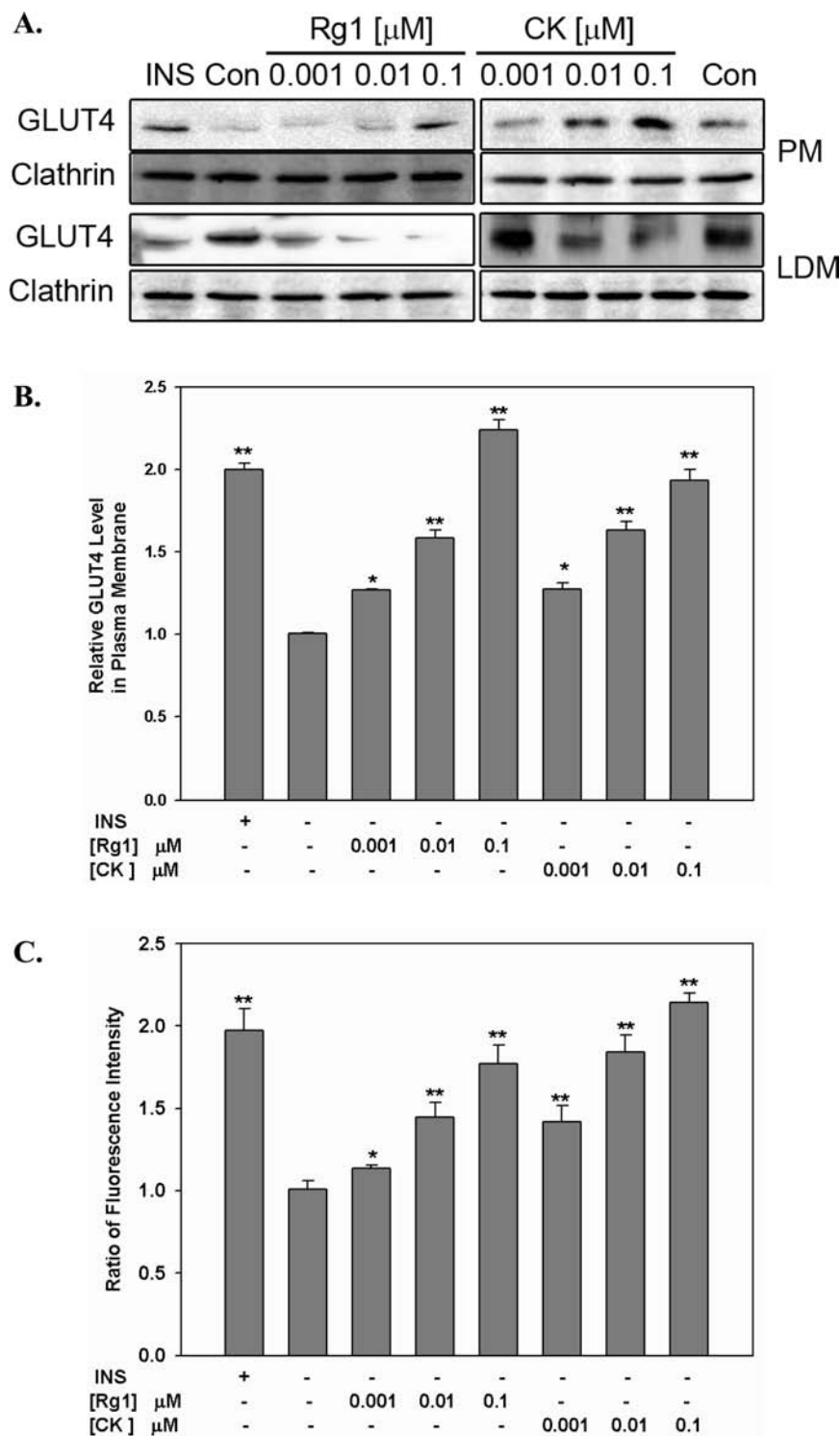


Figure 2. Effects of CK and Rg1 on GLUT4 translocation in 3T3-L1 adipocytes. Adipocytes were pretreated with or without CK and Rg1 at the indicated concentrations or 100 nM insulin (INS) alone for 2 h. (A) PM and LDM fractions were prepared as described under Materials and Methods and subjected to immunoblotting with antibodies against GLUT4 or clathrin, as indicated. (B) Quantitative analysis of relative level of GLUT4 from (A). Data represent mean \pm SE ($n = 3$). *, $p < 0.05$ versus the control. (C) Relative GLUT4 redistributed into plasma membrane was analyzed by indirect immunofluorescence with antibodies against HA-tagged GLUT4. Immunofluorescence intensity was detected at 480/575 nm. Data represent mean \pm SE ($n = 4-6$). *, $p < 0.05$ versus the control (Con).

the signaling pathways involved in CK- or Rg1-mediated activities, the effects of CK or Rg1 on glucose uptake and GLUT4 translocation were analyzed in the presence of specific inhibitors for PI3K and AMPK. Pretreatment of adipocytes with the PI3K inhibitor, LY294002, or AMPK inhibitor, compound C, markedly inhibited CK- and Rg1-mediated glucose uptake in 3T3-L1

adipocytes (49 or 41% of CK alone and 47 or 47% of Rg1 alone, respectively, $p < 0.05$, **Figure 6A**). Direct evidence for the activation of PI3K and AMPK signal pathways was provided by studying the phosphorylation of AKT and AMPK in CK- or Rg1-treated adipocytes. Results shown in **Figure 6B,C** demonstrate that both CK and Rg1 significantly increased the phosphorylation

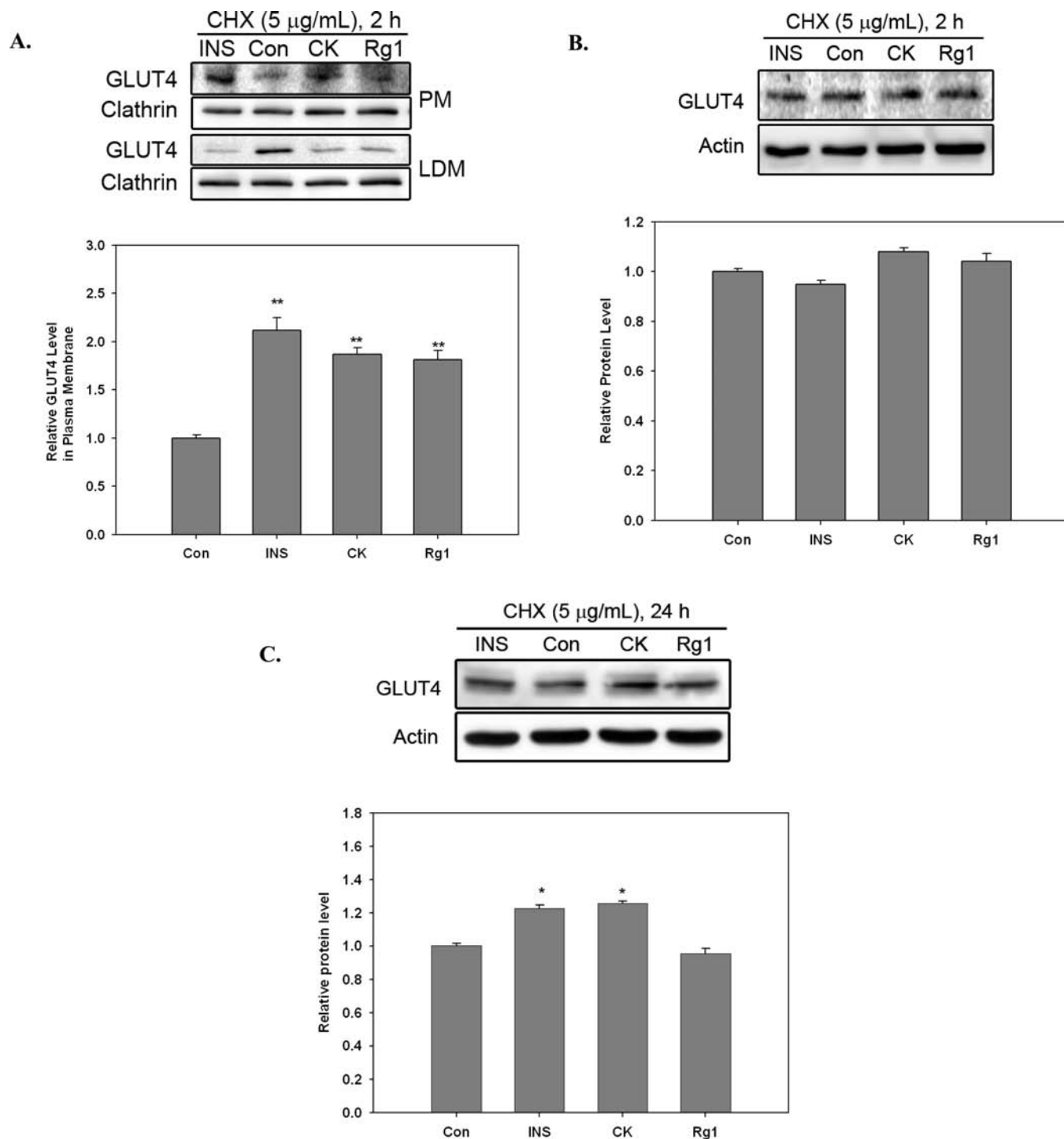


Figure 3. Effects of CHX on GLUT4 translocation and total GLUT4 protein level in CK- and Rg1-treated 3T3-L1 adipocytes. Adipocytes were pretreated with CHX (5 $\mu\text{g/mL}$) for 1 h and then in combination with or without 0.1 μM CK or Rg1 or 100 nM insulin (INS) for 2 or 24 h. PM and LDM fractions (**A**), total cell lysates of 2 h treatment (**B**), and 24 h treatment (**C**) were prepared as described under Materials and Methods and subjected to immunoblotting with antibodies against GLUT4, clathrin, or β -actin, as indicated. Data represent mean \pm SE ($n = 3$). *, $p < 0.05$ versus the control (Con).

of these kinase proteins (151 and 198% of control for pAKT; 175 and 173% of control for pAMPK, $p < 0.05$). Preincubation of adipocytes with LY294002 or compound C markedly suppressed the phosphorylation level of AKT or AMPK in CK- and Rg1-treated adipocytes (21 or 71% of CK alone and 46 or 65% of Rg1 alone, respectively, $p < 0.05$, **Figure 6B,C**). To assess the association of CK- and Rg1-induced membrane translocations of GLUT4 with the activation of PI3K and AMPK, the GLUT4 level in the PM fraction isolated from adipocytes pretreated with LY294002 or compound C was measured. As shown in **Figure 6D**, both

LY294002 and compound C prevented the translocation of GLUT4 from the cytosol to PM fraction induced by CK or Rg1 (38 and 36% of CK alone or 42 and 60% of Rg1 alone, respectively, $p < 0.05$, **Figure 6D**). These results suggest that ginsenosides CK or Rg1 stimulated glucose uptake and GLUT4 translocation by activating the PI3K and insulin-independent AMPK pathways.

DISCUSSION

Metabolic syndrome is a complex and chronic progressive syndrome that leads to adverse function of many organs. Among

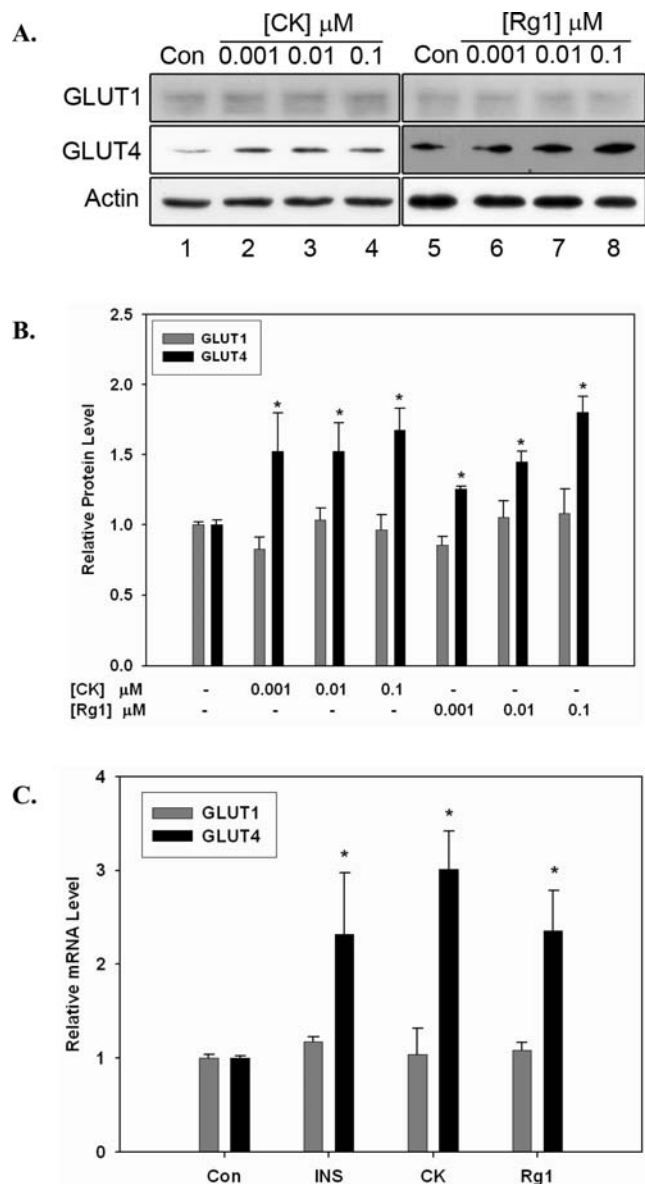


Figure 4. Effects of CK and Rg1 on GLUT1 and GLUT4 gene expression in 3T3-L1 adipocytes. Adipocytes were incubated with or without the indicated concentrations of CK and Rg1 or 100 nM of insulin (INS) for 24 h. (A) Representative immunoblots and (B) quantitation of GLUT1 and GLUT4 protein in total lysates. Adipocytes were treated with 0.1 μM CK or Rg1 or 100 nM insulin for 24 h. (C) GLUT1 and GLUT4 mRNA were quantified by Q-PCR. Relative mRNA level was calculated as fold increase of control. Data represent mean \pm SE ($n=3$). *, $p < 0.05$ versus the control (Con).

metabolic syndrome components, hyperglycemia was the main contributor of the associated disorders, which can exacerbate defective glucose disposal by interfering with insulin action in insulin-target tissues. Thus, regulation of glucose uptake is critical for treatment of metabolic syndrome as well as diabetes. Regulation of glucose transporter gene expression is an important mechanism in the regulation of glucose uptake in animal cells. Glucose transport in adipocytes is regulated by facilitative glucose transporters, GLUT1 and GLUT4 (14). GLUT1 is essentially for basal glucose transport in various tissues, whereas GLUT4 is expressed in insulin-responsive tissues, including adipose and skeletal muscle cells. The study of glucose uptake in adipocytes is important in that there is a strong correlation

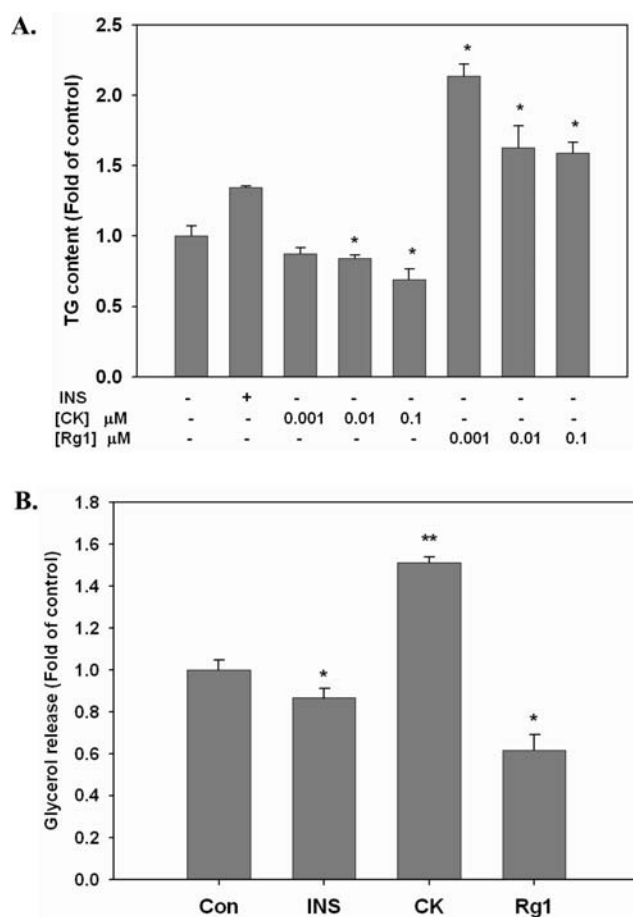


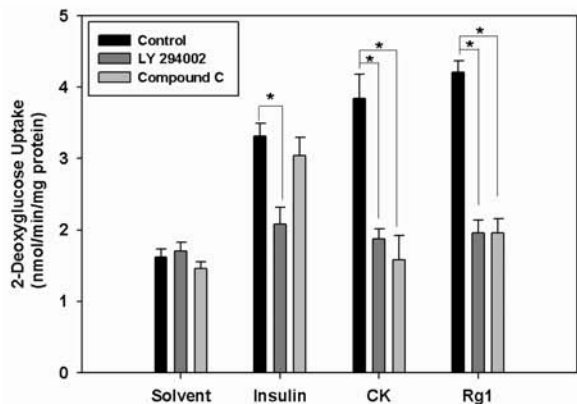
Figure 5. Effects of CK and Rg1 on TG accumulation and glycerol release in 3T3-L1 adipocytes. (A) Adipocytes were treated with indicated concentrations of CK or Rg1 for 48 h and subjected to assay for TG. (B) Adipocytes were treated with 100 nM insulin (INS) or 0.1 μM CK or Rg1 for 48 h, and the amount of extracellular glycerol was quantified as described under Materials and Methods. Data represent mean \pm SE ($n=4$). *, $p < 0.05$ versus the control (Con).

between abnormal fatty acid metabolism and the development of insulin resistance in muscle cells (15).

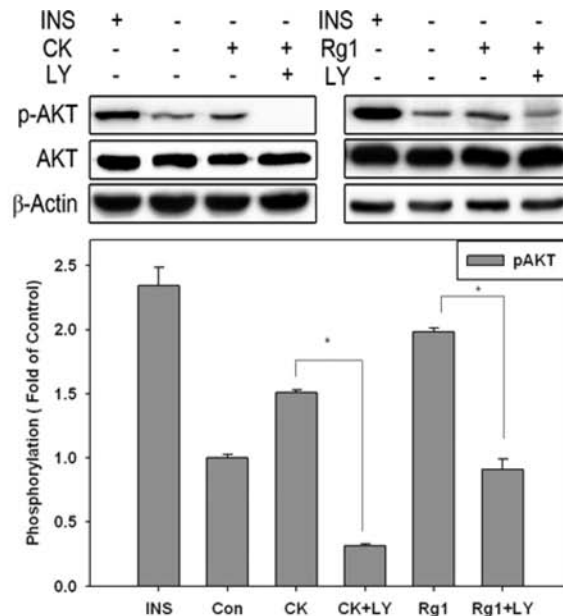
This study is designed to investigate the insulin-like activity of ginsenosides in facilitating glucose uptake into 3T3-L1 adipocytes. The hypoglycemic effect of ginseng is one of the most studied pharmacological activities of this traditional medicine. Although many in vitro and animal studies have repeatedly shown the hypoglycemic effects of ginseng, the efficacy evidence of ginseng is still inconclusive (16). A likely explanation for the variable hypoglycemic effects of different ginseng preparations is the marked difference in the profiles of ginsenosides (17). To avoid any ambiguous effect, purified ginsenosides CK and Rg1 were used in this study. There is no apparent cytotoxicity in these compounds at doses up to 10 μM toward the differentiated 3T3-L1 cells (data not shown). We used submicromolar concentrations (0.001–0.1 μM) of ginsenosides CK and Rg1 that are much lower than most of the studies for ginsenosides from different sources (18–20). The concentrations used in this study are important in that they could decrease the side effects or other unwanted activities.

In this study, we found that both ginsenosides significantly stimulated glucose uptake in 3T3-L1 adipocytes with a potency comparable to that of insulin. It has been shown that insulin-stimulated glucose uptake is mediated primarily by the rapid movement of GLUT4 from an intracellular compartment to the

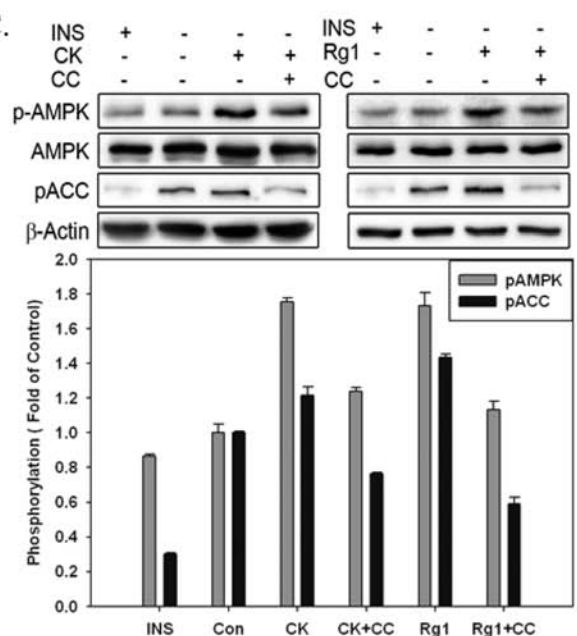
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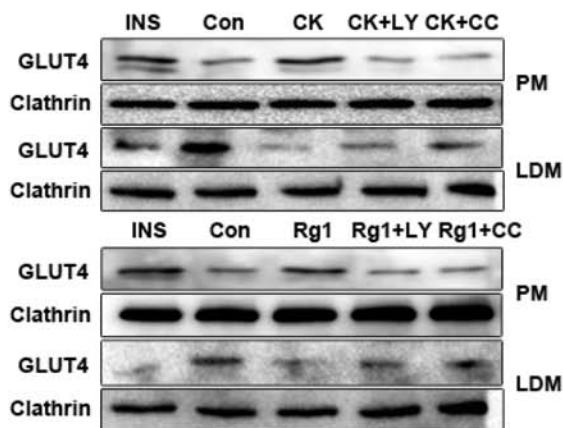
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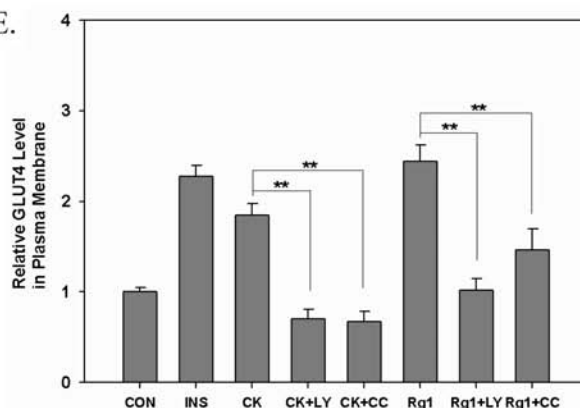


Figure 6. PI3K and AMPK pathways are involved in CK- and Rg1-induced glucose uptake and GLUT4 translocation. Adipocytes were incubated for 2 h with or without 0.1 μ M CK or Rg1 or 100 nM insulin (INS) alone or in combination with 1 μ M compound C (CC) or 1 μ M LY294002 (LY). The cells were then analyzed for 2-DG uptake (**A**). The phosphorylation level of AKT (**B**) or AMPK and ACC (**C**) with or without LY294002 or compound C was examined in CK- or Rg1-treated adipocytes. PM and LDM fractions were prepared and subjected to immunoblotting with antibodies against GLUT4 or clathrin (**D**). Panel **E** displays the results of quantitative analysis of relative level of GLUT4 from (**D**). Data represent mean \pm SE ($n = 3$). *, $p < 0.05$ versus the control (Con).

cell surface (1). In this study, we found that, in a manner very similar to insulin, both of CK and Rg1 induced significant increases in the translocation of GLUT4 protein from intracellular vesicle pool into the PM fraction. In addition to membrane fractionation, evidence of the CK- and Rg1-mediated membrane translocation was also confirmed using indirect immunofluorescence assay of the surface-exposed HA-GLUT4-GFP (12). By inhibition of protein synthesis by CHX, we further demonstrated that the CK- or Rg1-stimulated acute GLUT4 translocation in adipocytes is independent of de novo GLUT4 synthesis. Thus, our results showed that enhanced GLUT4 translocation is essential for the acute stimulation of glucose uptake in CK- or Rg1-treated adipocytes.

Overexpression of GLUT4 has been shown to improve insulin sensitivity, suggesting that GLUT4 is a potential therapeutic target for the treatment of insulin resistance (21). In addition, the effect of insulin is dependent not only on GLUT4 translocation but also on the total cellular content of the transporter; it is thus important for us to investigate the effect of CK or Rg1 on GLUT4 gene expression in adipocytes. Here, we demonstrate that prolonged exposure (24 h) of 3T3-L1 adipocytes with CK and Rg1 induced only the GLUT4 expression and showed little effect on GLUT1 expression. In addition, both the GLUT4 mRNA and protein were induced by CK and Rg1, suggesting that these ginsenosides may confer stimulation of GLUT4 gene transcription or stabilization of GLUT4 mRNA. Further study of the prolonged effect of ginsenosides in adipocytes in the presence of CHX suggested that stabilization of GLUT4 protein may also account, although in a lesser part, for the CK-mediated, but not Rg1-mediated, increase of GLUT4 protein level. As prolonged incubation of adipocytes with CK in the presence of CHX caused a small but significant increase (1.2-fold) in the total GLUT4 protein level, which is not found in Rg1-treated adipocytes. These results also suggest that the mechanisms of action of ginsenosides CK and Rg1 may not be completely identical. Specific induction of GLUT1 or GLUT4 gene expression has been shown in studies as the underlying mechanism of the enhanced glucose uptake induced by different compounds. Berberine was shown to activate GLUT1-mediated glucose uptake in 3T3-L1 adipocytes (22). TGF- β 1 has been shown to stimulate glucose uptake by enhancing GLUT1 expression in mesangial cells (23). Investigation of the hypoglycemic effect of ginsenoside Rb1 also demonstrated the specific induction of GLUT4 mRNA and protein level but not of GLUT1 expression in 3T3-L1 adipocytes (18). As the GLUT4 gene is subjected to complex tissue-specific and metabolic regulation, further studies are needed to understand the molecular mechanism of the CK- and Rg1-mediated induction of GLUT4 in adipocytes.

With increased intracellular glucose availability, we expected to observe increased adipogenesis in the treated adipocytes. Here, we showed that submicromolar concentrations of Rg1 significantly stimulated TG accumulation and inhibited lipolysis in adipocytes. As insulin is known to enhance lipogenesis and inhibit lipolysis in adipocytes, our results again showed the insulin-like activity of Rg1 in adipocytes. Differential effects of ginsenosides on adipogenesis in 3T3-L1 adipocytes have been reported. Several ginsenosides, including Rb1 and Rg1, at 20 μ M concentration, were shown to suppress triglyceride accumulation by activation of the PKA signaling pathway (19). However, studies have also indicated that Rb1 and other ginsenosides stimulate adipogenesis, which resulted in accumulation of TG in 3T3-L1 cells by enhancing the PPAR γ 2 and C/EBP α gene expression (18). Inhibition of lipolysis was also demonstrated in a study of the effect of ginseng extract in rat adipocytes (24). The discrepancies among these studies may result from distinct ginsenoside concentrations used.

Here, submicromolar concentrations were used throughout this study, which is much less than the doses of > 10 μ M ginsenosides used in previous studies on Rb1 or Rg1 (18, 19).

In this study, it is also interesting to note that ginsenoside CK displayed an opposite effect as it markedly suppressed TG accumulation and increased glycerol in 3T3-L1 adipocytes. Consistent with our findings, CK has been shown to attenuate hepatic lipid accumulation and enhance lipolysis via activation of AMPK in human hepatoma cells (20). Thus, we showed that ginsenosides CK and Rg1 exhibited an opposite effect on adipogenesis and lipolysis in adipocytes. Examples of the opposite effects of different ginsenosides were not uncommon and have been reported in studies of various physiological activities (3, 25, 26). Ginsenoside Rg1 was shown to stimulate nitric oxide release and enhance angiogenesis, whereas Rb1 exerted a significant inhibition on these effects (25). In our previous study, we also showed that CK and Rg1 exerted an opposite effect on glucose transport in human intestinal Caco-2 cells (9).

The stimulation of glucose uptake by insulin in adipocytes requires the insulin receptor-mediated tyrosine phosphorylation of IR substrate (IRS) family members and subsequent activation of PI3K and AKT (1). In addition to the PI3K pathway, AMPK has been shown to regulate glucose transport in muscle and adipocytes via an insulin-independent mechanism (27). In this study, inhibition of PI3K activity by LY294002 resulted in abrogation of glucose transport and GLUT4 translocation stimulated by CK, Rg1, and insulin. In addition, inhibition of AMPK by compound C blocked the CK- and Rg1-induced glucose transport and GLUT4 translocation but had no effect on the action of insulin. Furthermore, we also provide biochemical evidence to demonstrate that CK and Rg1 exerted insulin-like activities through activation of the PI3K and AMPK pathways. Herbal medicines have been shown to display significant effects in modulating glucose uptake in various experimental systems. Activation of PI3K has been known to be involved in the action of many natural compounds in facilitating glucose uptake (19, 28). Ginsenosides Rb1 and Re1 have been shown to display antidiabetic activities by increasing glucose uptake and membrane translocation of glucose transporters through the activation of PI3K activity (18). The pentacyclic ursane-type triterpenoid ursolic acid was shown to act as an insulin-mimetic in activating insulin receptor PI3K signaling pathways (28). AMPK is an important therapeutic target for regulating whole-body energy balance. Compounds that activate AMPK showed improved hypoglycemic effect by facilitating glucose uptake and GLUT4 translocations. It has been shown that many compounds or drugs with beneficial activity to metabolic syndrome are known to activate AMPK, with metformin as the prominent example (29, 30).

This study provides mechanistic explanations for the increased glucose uptake, GLUT4 translocation, and GLUT4 gene expression in CK- and Rg1-treated 3T3-L1 adipocytes. The effects of ginsenosides CK and Rg1 on GLUT4 translocation were shown to associate with activation of PI3K and AMPK signaling pathways. As one of the most popular traditional medicines used around the world, ginsenosides present promising candidates as insulin-like natural medicines. Despite the detailed mechanism of these compounds requiring further investigation, our findings provide evidence for two ginsenosides with marked insulin-like activity and potential in the treatment of metabolic syndrome or diabetes.

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