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Regulatory mechanism for the stimulatory action of genistein on glucose uptake *in vitro* and *in vivo*☆

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Abstract

Genistein, an isoflavone, is known to possess diverse biological functions such as antioxidative and anti-inflammatory actions. It also acts like estrogen and inhibits several tyrosine kinases. Genistein was reported to suppress insulin-mediated glucose uptake in adipocytes. In this study, we investigated the effects of genistein on glucose uptake *in vitro* and *in vivo* as well as the mechanisms associated with the glucose uptake. We found that genistein decreased nonfasting blood glucose levels in KK-Ay/Ta Jcl mice, a type 2 diabetic animal model. It also dose-dependently induced insulin secretion by Rin-5F cells. In L6 myotubes, it directly stimulated glucose uptake independently of insulin under normal and high glucose conditions in dose-dependent manners. It promoted the translocation of glucose transporter 4 to the cell membrane under both glucose conditions. Based on studies using inhibitors of signaling molecules related to glucose uptake, the stimulatory effect of genistein on glucose uptake appeared to be dependent on the phosphatidylinositol 3-kinase, mammalian target of rapamycin, protein kinase C and 5'-adenosine-monophosphate-activated protein kinase pathway under both glucose conditions. In addition, *O*-GlcNAcylation by *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate, an inhibitor of *N*-acetylglucosaminidase, reduced the stimulatory effect of genistein on glucose uptake under both glucose conditions. Taken together, genistein may regulate glucose uptake by increasing the phosphorylation and decreasing the *O*-GlcNAcylation of proteins related to glucose homeostasis.

Keywords: Genistein; Glucose uptake; GLUT4; *O*-GlcNAcylation

1. Introduction

Recently, evidence emerged that dietary phytoestrogens and isoflavones play beneficial roles in metabolic diseases such as obesity and diabetes as well as menopausal symptoms, osteoporosis, cancer and cardiovascular diseases [1–3].

Genistein, an isoflavone, is a major natural phytoestrogen found in soybeans. Considerable research attention has been focused on the high dietary intake of soy isoflavones because of their potentially beneficial effects associated with reduced risks of developing osteoporosis [4], high cholesterol [5], menopausal symptoms [6] and some forms of cancer [7]. The structure of genistein is similar to the primary structure of 17β-estradiol, which leads to its weak estrogenic agonist or antagonist activity that influences cell functions mediated through cytosolic/nuclear estrogen receptors (ER-α and ER-β) in genomic processes [8]. Emerging studies reported that administration of isoflavones or genistein lowered plasma glucose in diabetic animals [9–11], suggesting that genistein may be a plant-derived antidiabetic agent. However, the mechanism of genistein action in diabetes is unknown.

Insulin-stimulated glucose uptake by skeletal muscle plays an important role in the maintenance of whole-body glucose homeostasis. Glucose transporter 4 (GLUT4) is the main glucose transporter isoform expressed in skeletal muscle that mediates glucose uptake in response to hormones such as insulin, to stimuli such as exercise/contraction and hypoxia and to pharmacological interventions that alter mitochondrial energy output. Both insulin and exercise acutely stimulate glucose uptake by GLUT4 translocation to the cell surface of skeletal muscle cells [12,13]. The insulin signaling pathway to GLUT4 is triggered by activation of the insulin receptor tyrosine kinase, leading to tyrosine phosphorylation of insulin receptor substrate (IRS) proteins and their recruitment of phosphatidylinositol 3-kinase (PI3K). In turn, PI3K triggers the activation of protein kinase B (Akt)

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through the actions of two intermediate protein kinases, phosphoinositide-dependent kinase 1 and rictor/mammalian target of rapamycin (mTOR) [14,15]. Atypical protein kinase C (PKC) also acts as downstream target of PI3K to relay insulin signals for GLUT4 translocation [16]. Two cellular consequences of muscle contraction, a transient increase in the intracellular calcium concentration and an increase in the adenosine monophosphate (AMP)/adenosine triphosphate (ATP) ratio, are thought to contribute to enhanced GLUT4 translocation to the cell surface. The former signal is mediated through the activations of calcium/calmodulin-dependent protein kinase II and conventional PKC [17]. In addition, calcium/calmodulin appears to activate the 5'-AMP-activated protein kinase (AMPK) signaling pathway through the upstream kinases calcium/calmodulin-dependent protein kinase kinase α and β [18,19].

O-GlcNAcylation is a common posttranslational modification that adds a single *N*-acetylglucosamine (GlcNAc) moiety to serine or threonine residues in proteins by the activities of *O*-GlcNAc transferase and *N*-acetylglucosaminidase (*O*-GlcNAcase) [20–22]. Some reports have shown a correlation between the development of insulin resistance and increased *O*-GlcNAcylation of skeletal muscle proteins [23,24].

Based on these findings, the present study was conducted to investigate the effects of genistein on glucose uptake *in vitro* and *in vivo* using L6 myotubes and KK-Ay/Ta Jcl mice and to clarify the mechanisms associated with the enhanced glucose uptake.

2. Materials and methods

2.1. Materials

L6 myoblast cells derived from a rat and RIN-5F cells derived from rat pancreatic β-cells were purchased from American Type Culture Collection (Rockville, MD, USA; ATCC numbers: CRL-1458 and CRL-2058, respectively). Genistein was purchased from Tyger Scientific Inc. (Ewing, NJ, USA). The following items were purchased from the cited commercial sources: Glucose CII Test Kit, rapamycin and compound C from Wako Pure Chemical Industries Ltd. (Osaka, Japan); Lactate Dehydrogenase (LDH) Cytotoxicity Detection Kit from Takara Bio Inc. (Shiga, Japan); High Range Rat Insulin ELISA Kit from Mercodia AB (Uppsala, Sweden); 5-aminoimidazole-4-carboxamide 1-β-D-ribofuranoside (AICAR) and *O*-(2-acetamido-2-deoxy-D-glucopyranosylidene) amino *N*-phenyl carbamate (PUGNAc) from Toronto Research Chemicals (Toronto, ON, Canada); Pefabloc SC from Roche Applied Science (Indianapolis, IN, USA); Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS) and Gö6983 from Sigma (St. Louis, MO, USA); LY294002 from Merck4 Bioscience (Calbiochem, San Diego, CA, USA); anti-phospho-AMPK and anti-AMPK antibodies from Cell Signaling Technology (Beverly, MA, USA); anti-Na^+/K^+ ATPase α-1 antibody from Millipore (Billerica, MA, USA); anti-GLUT4 antibody from AbD Serotec (Oxford, UK); horseradish-peroxidase-conjugated anti-mouse and anti-rabbit IgG antibodies from Invitrogen (San Diego, CA, USA); ECL Plus Western blotting detection reagents and Hybond ECL nitrocellulose membrane from GE Healthcare (Buckinghamshire, UK).

2.2. Animals and diets

All animal experiments were conducted in accordance with the guidelines established by the Animal Care and Use Committee of Tokyo Noko University and were approved by this committee. To determine the effect of genistein on nonfasting blood glucose levels, KK-Ay/Ta Jcl mice were used as an animal model of type 2 diabetes [25].

Male KK-Ay/Ta Jcl and C57BL/6J Jcl mice (3 weeks of age) were obtained from CLEA Japan, Inc., Tokyo, Japan. The animals were individually housed in stainless-steel cages with wire bottoms in an air-conditioned room with a temperature of 22°C±2°C, a relative humidity of 60%±5% and an 08:00–20:00 h light cycle. All the mice were maintained on a stock CE-2 pellet diet (CLEA Japan, Inc.) for 3 days and thereafter on a basal 20% casein diet (20C) for 4 days [26]. The composition of the 20C diet was as follows (dry weight basis): 20% casein (Oriental Yeast Co., Tokyo, Japan); 7% corn oil (Hayashi Chemicals Co., Tokyo, Japan); 13.2% α-cornstarch (Nihon Nosan Kogyo Co., Yokohama, Japan); 49.75% β-cornstarch (Nihon Nosan Kogyo Co.); 3.5% mineral mixture (AIN-93G composition; Nihon Nosan Kogyo Co.); 1% vitamin mixture (AIN-93 composition; Nihon Nosan Kogyo Co.); 0.25% choline bitartrate (Wako Pure Chemical Industries Ltd.); 0.3% L-cystine (Wako Pure Chemical Industries Ltd.) and 5% cellulose powder (Oriental Yeast Co.). After preliminary feeding for 1 week, blood was collected from the tail vein at 10:00 h under the conditions that the mice were allowed free access to their diet and water. After bursting of the blood cells (5 μl) in water (45 μl), 20% (wt/vol) trichloroacetic acid aqueous solution (50 μl) was added, and the test tube containing the mixture was kept in ice-cold water. The mixture was then centrifuged at 13,000 *g* and 4°C for 5 min. The resultant supernatant (10 μl) was subjected to glucose determination using the Glucose CII Test Kit and measurement of the absorbance at 505 nm with a spectrophotometer (Model U-1100; Hitachi Science Systems Ltd., Ibaraki, Japan). Subsequently, the KK-Ay/Ta Jcl mice (now 6 weeks of age) were divided into two groups with similar nonfasting blood glucose levels and body weights (0 week). The KK-Ay/Ta Jcl mice in each of the two groups were given either the 20C diet as a diabetic control group or the 20C diet supplemented with 0.1% genistein as a genistein-fed diabetic group for 5 weeks. Genistein was supplemented to the 20C diet at the expense of the β-cornstarch. Likewise, the C57BL/6J mice were given the 20C diet as a nondiabetic group for 5 weeks. Water and each diet were always available, and blood was collected every week at 10:00 h to determine the nonfasting blood glucose levels as described above. Glucose excretion into urine was also determined every week using a commercial urine test paper (URIACE-M, Terumo Corporation, Tokyo, Japan). At the end of feeding period, blood was collected at 10:00 h from the tail vein, followed by exsanguinations from the heart under anesthesia with Nembutal (Dainippon Sumitomo Pharma Co., Ltd., Osaka, Japan).

2.3. Culture of L6 myoblast and RIN-5F cells

L6 myoblast cells were cultured in DMEM containing 10% (vol/vol) FBS, penicillin G (100 U/ml) and streptomycin (100 μg/ml) in a humidified 5% CO_2 incubator at 37°C. To differentiate to myotubes, the myoblast cells (5×10^4 or 7×10^5) were seeded in Falcon 24-place multiwell plates or 60-mm culture dishes and cultured to 90% confluency in DMEM containing 2% FBS for 1 week. RIN-5F cells were maintained in RPMI 1640 supplemented with 10% (vol/vol) FBS, streptomycin (100 μg/ml) and penicillin G (100 U/ml) (10% FBS/RPMI 1640) under an atmosphere of 5% CO_2/95% humidified air at 37°C. The medium was renewed every 3 days.

2.4. Insulin secretion and cytotoxicity test of cultured RIN-5F cells

The effect of genistein on insulin secretion was examined using a previously described procedure [27] with slight modifications. Briefly, the RIN-5F cells were subcultured into Nunc 24-place multiwell plates at 2.5×10^5 cells/well. After culture in 1 ml of 10% FBS/RPMI 1640 for 72 h, the medium in each well was removed, and the cells were washed once with Ca^{2+}-/Mg^{2+}-free phosphate-buffered saline. Thereafter, the RIN-5F cells were cultured in 1 ml of fresh 1% FBS/RPMI 1640 with or without genistein for 3 h. Aliquots of the culture media from all the wells were centrifuged, and the insulin concentration of each supernatant (10 μl aliquot) was determined by the absorbance at 450 nm using the High Range Rat Insulin ELISA Kit and the Model 680 microplate reader.

To carry out cytotoxicity tests, RIN-5F cells were cultured in 1% FBS/RPMI 1640 with or without 0–100 μM genistein for 3 h as described above. As a positive control, RIN-5F cells were cultured in 1% FBS/RPMI 1640 containing 2% (vol/vol) Triton X-100 for 3 h. At the end of the culture, aliquots of the culture media from all the wells were centrifuged, and the LDH activity of each supernatant was determined by the absorbance at 490 nm using the LDH Cytotoxicity Detection Kit and a microplate reader (Model 680; Bio-Rad Laboratories, Hercules, CA, USA). The mean value for media containing Triton X-100 was regarded as the maximum cytotoxicity (100%).

2.5. Determination of glucose uptake by cultured L6 myotubes

Briefly, perfused L6 myoblast cells were subcultured into Falcon 24-place multiwell plates at 5×10^4 cells/well and grown for 11 days in 0.4 ml of 2% FBS/DMEM to allow the formation of myotubes. The medium was renewed every 2 days. Subsequently, the 11-day-old myotubes were incubated in filter-sterilized Krebs–Henseleit buffer (141 mg/L $MgSO_4$, 160 mg/L KH_2PO_4, 350 mg/L KCl, 6900 mg/L NaCl, 373 mg/L $CaCl_2\cdot2H_2O$, 2100 mg/L $NaHCO_3$, pH 7.4) containing 0.1% bovine serum albumin, 10 mM Hepes and 2 mM sodium pyruvate (KHH buffer) for 2 h. The myotubes were then cultured for 4 h in KHH buffer containing 11 mM glucose with or without genistein (10–100 μM) and with or without various inhibitors as follows: 10 μM LY 294002, a potent PI3K inhibitor; 10 μM rapamycin, an mTOR inhibitor; 5 μM Gö6983, a broad PKC inhibitor; 5 μM compound C, an AMPK inhibitor; 50 μM PUGNAc, an *O*-GlcNAcase inhibitor. The differences in the glucose concentrations in the KHH buffer before and after culture were determined by the absorbance at 505 nm using a microplate reader (Model AD200; Beckman Coulter, Brea, CA, USA) and the Glucose CII Test Kit. The amounts of glucose consumed were calculated.

2.6. Subcellular fractionation and GLUT4 translocation analysis

To prepare plasma membrane and postplasma membrane fractions, we carried out a rapid plasma membrane preparation method as described previously [28]. Briefly, L6 myotubes were harvested with buffer A (50 mM Tris-HCl pH 8.0, 0.5 mM dithiothreitol) containing 0.1% Nonidet P-40 (NP-40), protease inhibitors (1 mM phenylmethylsulfonyl fluoride and 0.2 mg/ml Pefabloc SC) and a phosphatase inhibitor (1 mM Na_3VO_4) and homogenized by 10 passages through a 30-gauge syringe needle. Each homogenate was centrifuged at 1000 *g* for 10 min at 4°C, and the pellet was resuspended in NP-40-free buffer A containing the same protease and phosphatase inhibitors. After incubation on ice for 10 min with occasional mixing, the samples were recentrifuged at 1000 *g* for 10 min at 4°C. The pellet was resuspended in buffer A containing 1% (vol/vol) NP-40 and the same protease and phosphatase inhibitors, incubated on ice for 1 h with occasional mixing and centrifuged at 16,000 *g* for 20 min at 4°C. The supernatant was collected as the plasma membrane fraction and stored at −80°C until analysis. The supernatants from the first and second centrifugations at

1000 *g* were pooled and centrifuged at 16,000 *g* for 20 min at 4°C. The supernatant was collected as the postplasma membrane fraction and stored at −80°C until analysis.

2.7. Western blot analysis

L6 myotubes were solubilized in a lysis buffer [10 mM Tris-HCl pH 7.4, 150 mM NaCl, 1% NP-40, 0.5% sodium deoxycholate, 0.1% sodium dodecyl sulfate (SDS), 0.5 mM dithiothreitol, 0.2 mg/ml Pefabloc SC, 1 mM Na_3VO_4] for 30 min at 4°C. The lysates were then sonicated for 10 s and centrifuged at 12,000 *g* for 15 min at 4°C. The protein concentrations of the supernatants were evaluated using a protein assay reagent (Bio-Rad Laboratories). Equal amounts of protein (20 μg/lane) and prestained molecular weight markers (Wako Pure Chemical Industries Ltd.) were loaded onto 10% premade polyacrylamide gels (Wako Pure Chemical Industries Ltd.), separated by electrophoresis and transferred to nitrocellulose membranes. The membranes were then incubated in a blocking solution comprising 3% BSA in Tris-buffered saline (TBS) for 1 h. After the incubation, the membranes were washed in TBS and incubated with anti-phospho-AMPK, anti-AMPK, anti-Na^+/K^+ ATPase α-1 or anti-GLUT4 antibodies overnight at 4°C. The membranes were then washed in TBS containing 0.1% (vol/vol) Tween-20 for 30 min and incubated with horseradish-peroxidase-conjugated anti-mouse or anti-rabbit IgG antibodies at a dilution of 1:5000 for 60 min at room temperature. Immunoreactive bands were detected using ECL Plus Western blotting detection reagents. The intensity of each band was analyzed with a lumino-image analyzer (Model LAS-4000 Mini; Fujifilm, Tokyo, Japan) coupled with image analysis software (Multi Gauge Ver. 3.0; Fujifilm).

2.8. Statistical analysis

All data are presented as the mean±S.E.M. The data were evaluated by a one-way analysis of variance. Differences between the mean values were assessed using Dunnett's multiple comparisons test. Statistical significance was considered for values of $P < .05$.

3. Results

3.1. Genistein suppresses the nonfasting blood glucose level in KK-Ay/Ta Jcl mice

To investigate the *in vivo* effect of genistein, we employed KK-Ay/Ta Jcl mice, a type 2 diabetic animal model. Fig. 1 shows the effect of genistein on the nonfasting blood glucose level. The blood glucose level gradually and almost linearly increased in diabetic mice for 3 weeks and maintained high values thereafter, while it remained unchanged and constant in C57BL/6 nondiabetic mice. Genistein (0.1% in the diet) significantly suppressed the blood glucose level after 1 and 5 weeks of feeding. As shown in Table 1, the food intake of the genistein-fed diabetic group for 5 weeks did not differ significantly from that of the diabetic group, suggesting that the suppressive effect of genistein on the nonfasting blood glucose level was not caused by reduced food intake but instead by its pharmacological action. There were slight decreases in the serum lipid levels such as total cholesterol, triglyceride and thiobarbituric acid reactive substances (TBARS) by genistein treatment.

3.2. Genistein delays the increase in urinary glucose excretion in proportion to increasing blood glucose

To investigate renal glucose excretion as a function of the blood glucose concentration in KK-Ay/Ta Jcl mice, we measured the urinary glucose concentration simultaneously with the blood glucose concentration. The diabetic group showed a more acute increase in glucose excretion in proportion to the nonfasting blood glucose level than the genistein-fed diabetic group. The increase in the genistein-fed diabetic group was delayed by about 2 weeks (Fig. 2).

3.3. Genistein stimulates glucose uptake in cultured L6 myotubes

Next, we determined the effect of genistein on glucose uptake under normal glucose (5.5 mM) and high glucose (25 mM) conditions, mimicking the normoglycemic condition and the hyperglycemic condition in diabetes, respectively. First, we examined glucose uptake in cultured L6 myotubes under normal and high glucose conditions. Independently of insulin, genistein dose-dependently and significantly stimulated glucose uptake at concentrations of 10–50 μM under the normal glucose condition (Fig. 3A). Under the high glucose condition, the maximum increase in glucose uptake was observed at 30 μM (Fig. 3B). Based on these results, we adopted 50 μM for normal and 30 μM for high glucose conditions as the optimal genistein concentrations, respectively, in the following experiments.

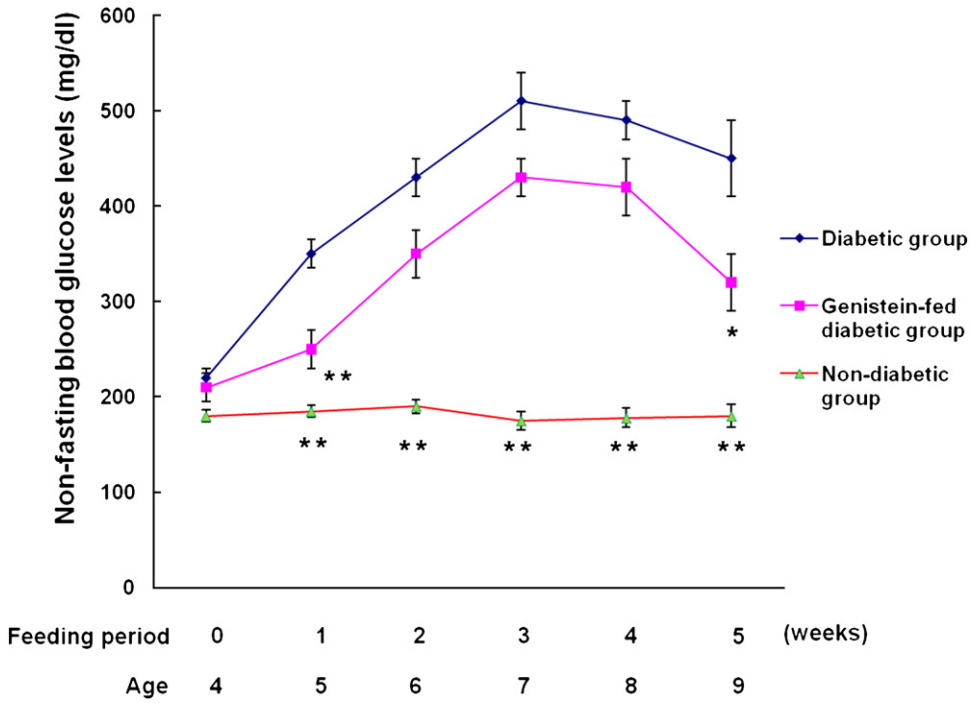


Fig. 1. Effect of genistein on nonfasting blood glucose levels in KK-Ay/Ta Jcl mice. Mice were maintained on diets and subjected to blood collection from the tail vein. Each value represents the mean±S.E.M. for six mice. *$P < .05$, **$P < .01$, significant difference vs. the diabetic group by Dunnett's multiple comparisons test.

Table 1
Metabolic characteristics of mice fed a standard diet (nondiabetic, diabetic) or a genistein-containing diet for 5 weeks

Measurement	C57BL/6	KK-Ay/Ta Jcl	
	Nondiabetic group	Diabetic group	Genistein-fed diabetic group
Initial body weight (g/mouse)	15.8±0.4*	18.1±0.5	18.2±0.6
Food intake (g/35 days)	130.8±3.7*	229.7±6.3	222.4±7.0
Body weight gain (g/35 days)	7.9±0.4*	18.6±0.9	19.2 ±0.6
Initial fasting blood glucose (mg/dl)	192.4±5.6*	218.5±10.1	215.6 ±6.8
Serum lipid levels			
Total cholesterol (mg/dl)	70.40±3.45*	111.22±7.49	101.56±6.37
Triglyceride (mg/dl)	73.57±4.44*	121.3±6.81	113.76±9.22
TBARS (mg/dl)	31.29±4.16*	50.05±1.96	47.29±5.55
Serum adiponectin (mg/ml)	13.18±0.44*	7.67±0.42	8.61±0.35
Serum insulin (ng/ml)	0.98±0.02*	13.25±2.35	19.66±2.38
Liver lipid levels			
Cholesterol (mg/g liver)	23.32±0.97*	37.48±1.78	37.48±1.81
Triglyceride (mg/g liver)	16.08±1.26*	29.26±2.12	27.89±1.66

Each value represents the mean±S.E.M. of six mice. *P <.05, significant difference vs. the diabetic group by Dunnett's multiple comparisons test.

3.4. Genistein induces translocation of GLUT4 to the plasma membrane

To investigate the effect of genistein on the activity of GLUT4, a main glucose transporter in skeletal muscle, we examined the translocation of GLUT4 to the plasma membrane (Fig. 4). Genistein directly stimulated GLUT4 translocation to the plasma membrane in cultured L6 myotubes under the normal and high glucose conditions. Increases in the GLUT4 translocation under the high glucose condition were observed twice at around 10 and 60 min, when calculated with image analysis software.

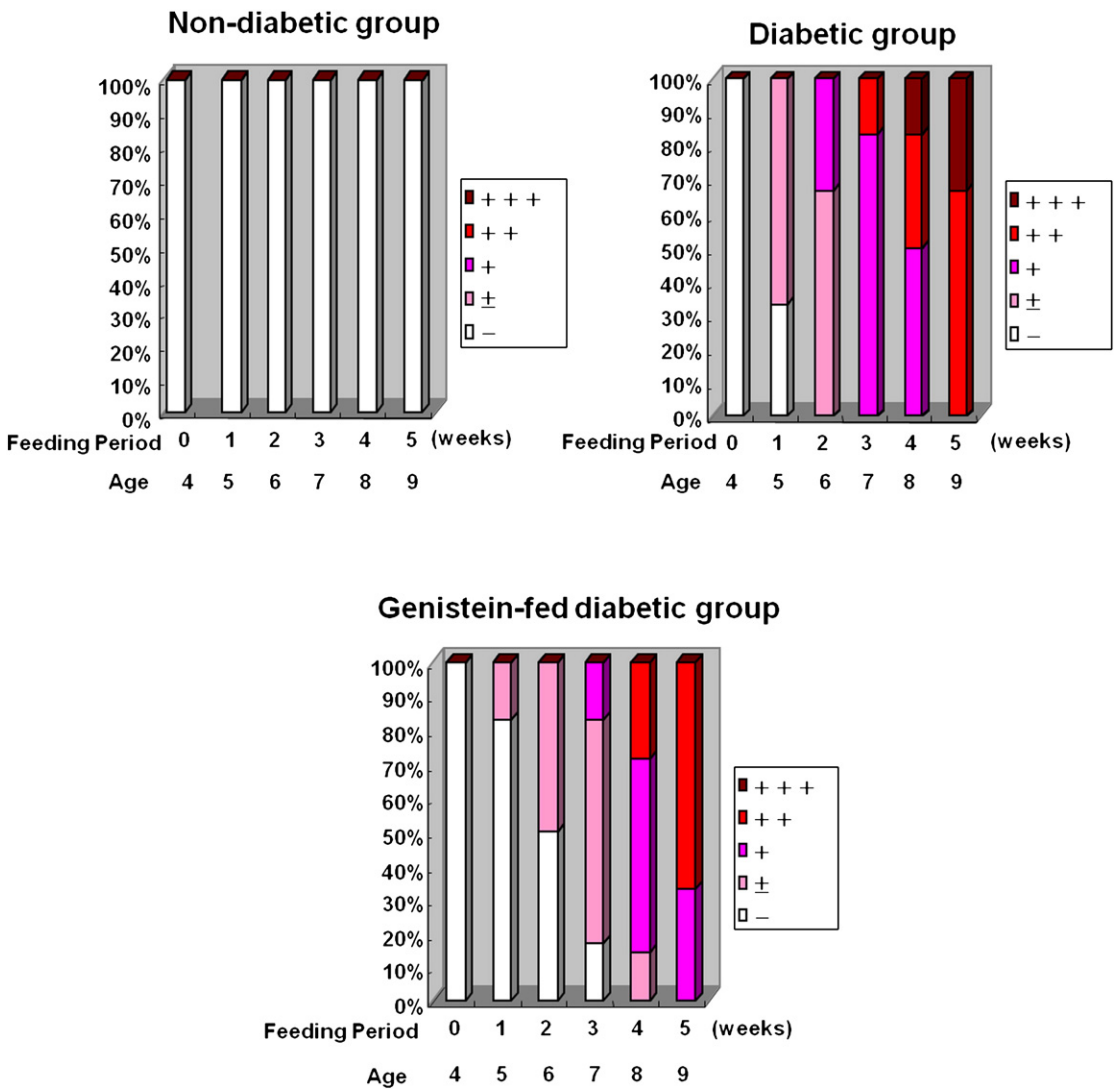


Fig. 2. Effect of genistein in urinary glucose excretion in KK-Ay/Ta Jcl mice. Urinary glucose excretion in mice was determined using urine test paper. Each group consisted of six mice. Urine glucose concentrations: − (~0 mg/dl); ± (~50 mg/dl); + (~100 mg/dl); ++ (~500 mg/dl); +++ (~2000 mg/dl).

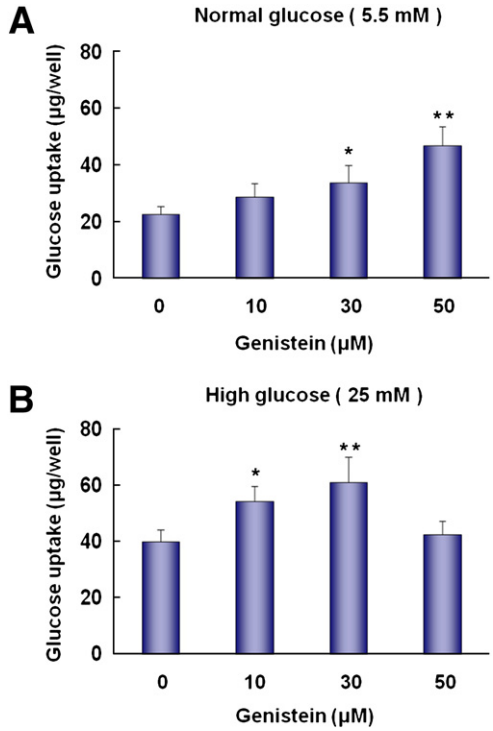


Fig. 3. Effect of genistein on glucose uptake in cultured L6 myotubes under normal and high glucose conditions. L6 myotubes were preincubated in KHH buffer without glucose for 2 h. They were then incubated in KHH buffer containing 11 mM glucose and 10, 30 or 50 mM genistein for 4 h, and the glucose uptake was determined. Each value represents the mean±S.E.M. for six wells. *$P < .05$, **$P < .01$, significant difference vs. the control (0 μM genistein) group by Dunnett's multiple comparisons test.

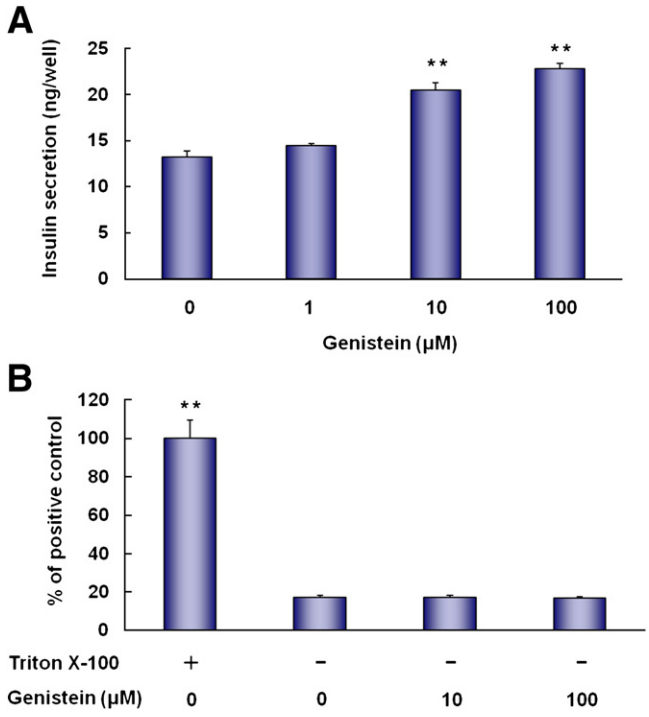


Fig. 5. Effect of genistein on insulin secretion from and viability of cultured RIN-5F cells. Each value represents the mean±S.E.M. for six wells. **$P < .01$, significant difference vs. the control (0 μM genistein) group by Dunnett's multiple comparisons test.

These results suggest that genistein induced the increase in glucose uptake by increasing the translocation of GLUT4 to the plasma membrane.

3.5. Genistein stimulates insulin secretion by RIN-5F cells

To investigate the effect of genistein on insulin secretion, we examined insulin secretion by RIN-5F cells, a rat islet tumor cell line. Genistein dose-dependently stimulated insulin secretion, and significant effects were seen at 10 and 100 μM (Fig. 5A). As shown in Fig. 5B, the cytotoxicity of genistein at 10 and 100 μM did not increase as

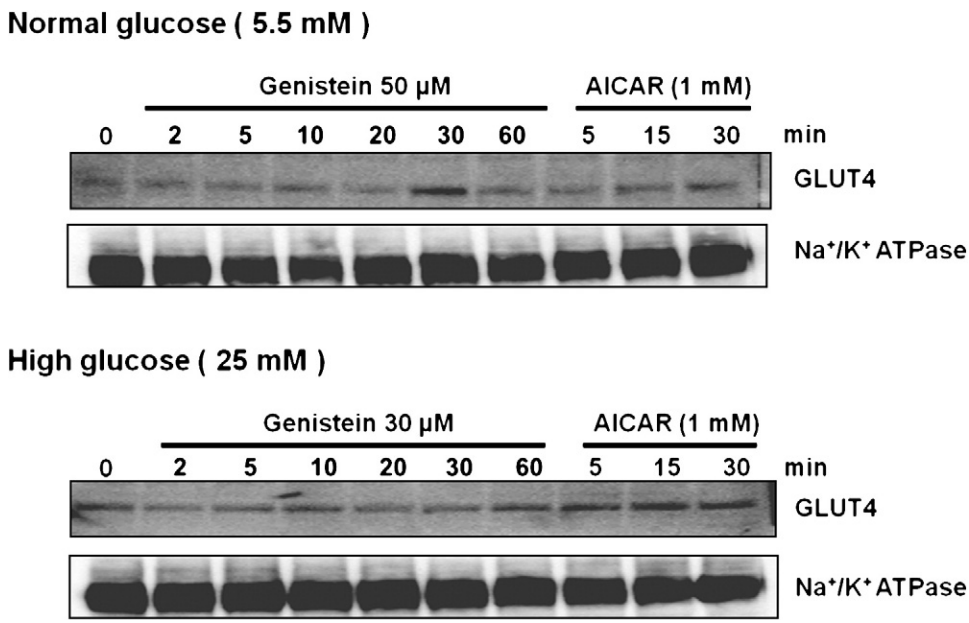
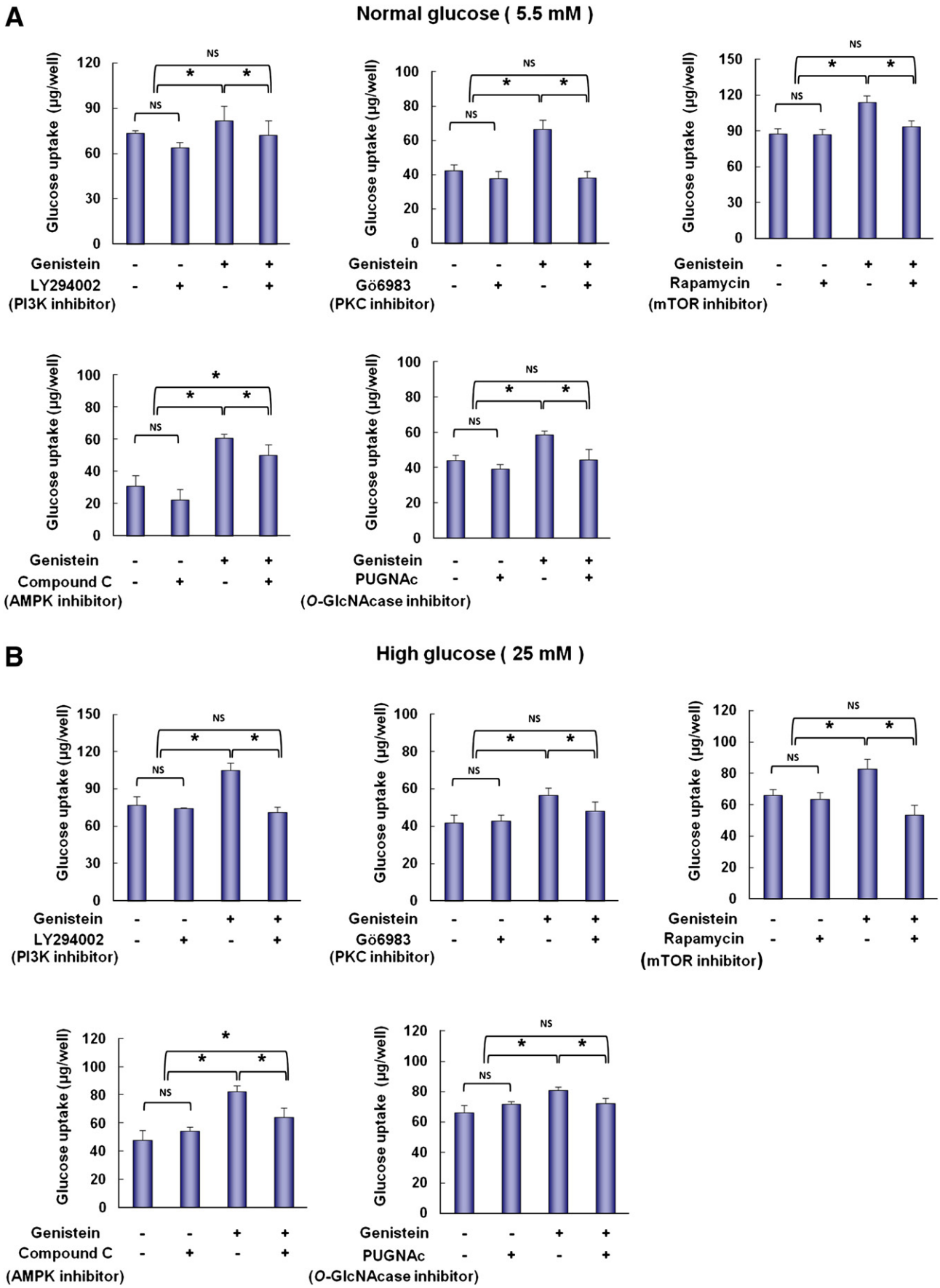


Fig. 4. Genistein promotes GLUT4 translocation in L6 myotubes under normal and high glucose conditions. L6 myotubes were incubated for 2, 5, 10, 20, 30 or 60 min in KHH buffer containing 30 or 50 mM genistein with 11 mM glucose and 1 mM AICAR for 5, 15 or 30 min. Subcellular membrane fractions of the L6 myotubes were prepared by a rapid plasma membrane preparation method. The plasma membrane fractions (20 μg) were subjected to SDS–polyacrylamide gel electrophoresis and Western blotting analyses using anti-GLUT4 and anti-Na^+/K^+ ATPase antibodies.


A
Normal glucose (5.5 mM)
Glucose uptake (μg/well)
NS
*
Genistein
LY294002
(PI3K inhibitor)
Gö6983
(PKC inhibitor)
Rapamycin
(mTOR inhibitor)
Compound C
(AMPK inhibitor)
PUGNAc
(O-GlcNAcase inhibitor)
B
High glucose (25 mM)

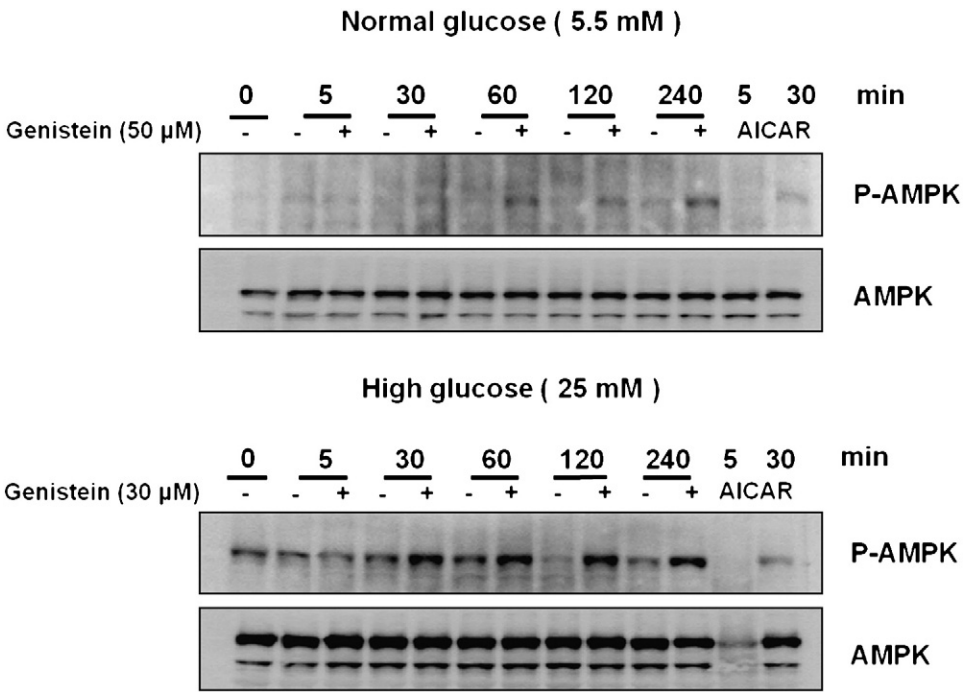


Fig. 7. Effect of genistein on the phosphorylation of AMPK. L6 myotubes were preincubated in Krebs–Henseleit buffer without glucose for 2 h. They were then incubated in KHH buffer containing 11 mM glucose in the presence or absence of 30 or 50 μM genistein for 5, 30, 60, 120 or 240 min and 1 mM AICAR for 5 or 30 min. Total lysates were analyzed by immunoblotting with anti-phospho-AMPK and anti-AMPK antibodies.

compared with the control (0 μM genistein), indicating that the stimulatory effect of genistein on insulin secretion was not caused by its cytotoxicity.

3.6. Stimulatory effect of genistein on glucose uptake is dependent on the PI3K, PKC, mTOR and AMPK pathways and also is inhibited by the induction of O-GlcNAcylation

To determine the regulatory mechanism by which genistein induced the glucose uptake in L6 myotubes under normal and high glucose conditions, we performed glucose uptake assays using several kinase inhibitors, namely, LY 294002, a specific inhibitor of PI3K; Gö6983, a broad inhibitor of PKC; rapamycin, an inhibitor of mTOR and compound C, an ATP-competitive inhibitor of AMPK. The promotion of glucose uptake by genistein was completely inhibited by the treatments with LY294002, Gö6983, rapamycin and compound C (Fig. 6A and B). These results suggest that the stimulatory effect of genistein on glucose uptake is dependent on the PI3K, PKC, mTOR and AMPK pathways. And also to investigate the relationship between the increase in *O*-GlcNAcylation of nucleocytoplasmic proteins and the stimulatory effect of genistein on glucose uptake, we examined the effect of PUGNAc, an inhibitor of *O*-GlcNAcase, on the stimulation of glucose uptake by genistein. Increased *O*-GlcNAcylation by PUGNAc reduced the stimulatory effect of genistein on glucose uptake under both glucose conditions (Fig. 6). These findings suggest that genistein may induce the phosphorylation of *O*-GlcNAcylated signaling molecules related to glucose homeostasis through the removal of *O*-GlcNAcylation at serine/threonine residues.

3.7. Genistein induces the phosphorylation of AMPK

To examine the activity of AMPK, a well-known main regulator of glucose uptake in skeletal muscle cells, we investigated the temporal expression of phosphorylated AMPK. Genistein time-dependently stimulated the phosphorylation of AMPK under normal and high glucose conditions (Fig. 7). Consequently, these data suggest that the main mechanism of glucose uptake by genistein is mediated by the AMPK pathway.

4. Discussion

Genistein has attracted attention because of its apparent protective effects against cardiovascular disease, metabolic syndrome, osteoporosis and cancer [29,30]. The effects of isolated isoflavones including genistein on glucose and insulin sensitivity in humans are inconclusive at the present time because previous studies have used mixed isoflavones or phytoestrogens with different dosage regimens, soy foods or supplements. The hypoglycemic effects of soybean components such as genistein and daidzein have been widely studied in diverse diabetic animal models [9–11]. Park et al. [10] showed that genistein and daidzein modulate hepatic glucose and lipid regulatory enzyme activities in *db*/*db* mice. In the present study, we confirmed the antihyperglycemic activity of genistein in KK-Ay/Ta Jcl mice, a type 2 diabetic animal model, and clarified the regulatory mechanism of glucose uptake by genistein such as GLUT4 translocation and AMPK activation in cultured myotubes under normal and high glucose conditions. KKAy/Ta Jcl mice have been widely used as diabetic model animals

Fig. 6. Effects of various inhibitors on genistein-promoted glucose uptake under normal (A) and high (B) glucose conditions. L6 myotubes were preincubated in KHH buffer without glucose for 2 h. They were then incubated for 4 h in Krebs–Henseleit buffer with 11 mM glucose in the presence or absence of 30 or 50 μM genistein and 10 μM LY 294002, 5 μM Gö6983, 10 μM rapamycin, 5 μM compound C or 50 μM PUGNAc. Each value represents the mean±S.E.M. for six wells. Statistical significance is indicated in the figure: $*P < .05$. NS, not significant.

that rapidly develop peripheral glomerular capillary basement membrane thickening in the kidney, resulting in severe hyperinsulinemia and hyperglycemia [25]. As expected, KKAy/Ta Jcl mice were found to rapidly develop hyperglycemia. Nevertheless, genistein significantly suppressed the increase in the nonfasting blood glucose level compared with the diabetic group after 1 and 5 weeks of feeding. We also found a delay in renal glucose secretion in the genistein-fed diabetic group compared with the diabetic group. These findings support the antihyperglycemic activity of genistein. Our data further revealed that genistein induced glucose uptake by increasing GLUT4 translocation under both normal and high glucose conditions. We observed that genistein stimulated the insulin secretion without any cytotoxicity at concentrations of up to 100 μM in RIN-5F cells. These observations suggest that the stimulatory effect of genistein on glucose uptake and insulin secretion was not caused by cytotoxicity and that it has a selective effect on glucose homeostasis. These findings are consistent with previous reports that genistein acutely stimulates insulin secretion by pancreatic β-cells [31] and that genistein derivatives stimulate glucose uptake in L6 myotubes [32].

Glucose uptake in skeletal muscle is primarily mediated by GLUT4. The number of GLUT4 molecules at the surface of muscle cells increases rapidly in response to insulin, contraction, depolarization or energy deprivation. However, distinct mechanisms underlie the increased expression of surface GLUT4 in each case. Insulin promotes its exocytosis to the membrane through diverse signal cascades including insulin receptor, PI3K, PKC, Akt and mTOR. In contrast, muscle contraction and energy demand reduce GLUT4 endocytosis. Based on these signaling pathways related to glucose uptake, we investigated the signaling pathways for glucose uptake by genistein using diverse kinase inhibitors. We found that the stimulatory effect of genistein on glucose uptake appeared to be dependent on the PI3K, PKC, mTOR and AMPK pathway under normal and high glucose conditions.

O-GlcNAcylation is a process that adds a single GlcNAc moiety to serine or threonine residues in proteins. It may play a role in certain disease states such as Parkinson's disease, Alzheimer's disease and diabetes [33]. In patients with diabetes, sustained hyperglycemia leads to insulin resistance (glucose toxicity) [34]. Enhanced *O*-GlcNAc modification of proteins related to glucose homeostasis may affect the insulin response. For instance, impaired insulin signaling concomitant with increased *O*-GlcNAcylation of IRS-1 and IRS-2 was reported in skeletal muscle of rats infused with glucosamine plus insulin [35], and enhanced *O*-GlcNAc modification of IRS-1 and β-catenin was observed in 3T3-L1 adipocytes rendered insulin resistance by treatment with an *O*-GlcNAcase inhibitor [36]. We found that the stimulation of glucose uptake by genistein was completely inhibited by PUGNAc, an *O*-GlcNAcase inhibitor, which led to an increase in *O*-GlcNAcylation of nucleocytoplasmic proteins. This observation suggests that genistein may phosphorylate its target proteins through the removal of *O*-GlcNAcylation at serine/threonine residues of proteins participating in the glucose uptake process.

5'-Adenosine-monophosphate-activated protein kinase plays a central role in the regulation of glucose and lipid metabolism as an intracellular energy sensor. Upon activation by allosteric binding of AMP or phosphorylation at Thr172 of its catalytic subunit, AMPK accelerates ATP-generating catabolic pathways, including glucose uptake and glucose and fatty acid oxidation [37]. In many reports, AMPK activators such as AICAR, peroxisome proliferator-activated receptor γ agonists, metformin and berberine were shown to stimulate muscle glucose uptake in both cells and humans [38–40]. A recent report about AMPK activation, GLUT4 and GLUT1 expressions and PTP1B inhibition by genistein derivatives supported the notion that the activation of AMPK by genistein was involved in the mechanism for stimulation of glucose uptake [32].

In conclusion, the results of the present study suggest that genistein has an antihyperglycemic effect and that this effect is mediated through a main mechanism including the activation of AMPK and induction of GLUT4 translocation.

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