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Glyceollins, One of the Phytoalexins Derived from Soybeans under Fungal Stress, Enhance Insulin Sensitivity and Exert Insulinotropic Actions

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Glyceollins are a category of phytoalexins that are produced by soybeans under fungal stress, but their effects on glucose homeostasis remain unknown. We hypothesized that glyceollins play an important role in glucose homeostasis by regulating glucose utilization in adipocytes and improving β-cell function and survival. Glyceollins improved insulin-stimulated glucose uptake in 3T3-L1 adipocytes without activating the peroxisome proliferator-activated receptor-γ agonist. They decreased triacylglycerol accumulation in adipocytes. In addition, glyceollins slightly improved glucose-stimulated insulin secretion without palmitate treatment in Min6 cells, and they potentiated insulinotropic actions when 500 μM palmitate was used to induce β-cell dysfunction. This was associated with decreased β-cell apoptosis because of the attenuation of endoplasmic reticulum stress, as determined by mRNA levels of XBP-1, ATF-4, ATF-6, and CHOP. Glyceollins also potentiated GLP-1 secretion to enhance insulinotropic actions in enteroendocrine cells. In conclusion, glyceollins help normalize glucose homeostasis by potentiating β-cell function and survival and improving glucose utilization in adipocytes.

KEYWORDS: Glucose uptake; peroxisome proliferator-activated receptor-γ; insulin secretion; β-cell apoptosis; glucagon-like peptide-1; ER stress

INTRODUCTION

Type 2 diabetes emerges from uncompensated peripheral insulin resistance that is associated with unregulated nutrient homeostasis, obesity, and peripheral insulin resistance and progressive β-cell failure (*1*). Previous studies on experimental animals have shown that the failure of insulin secretion causes the development of type 2 diabetes, which is associated with decreased β-cell expansion (*2*). Thus, the development or the progression of type 2 diabetes can be prevented or delayed by promoting glucose-stimulated insulin secretion (GSIS) as well as by intervening in the onset of insulin resistance. Several plants and their components aimed at alleviating insulin resistance through activating the peroxisome proliferator-activated receptor-γ (PPAR-γ) and improving GSIS have been developed as diabetic drugs and functional foods (*3*, *4*).

Soybeans (*Glycine max* Merill) have long been consumed as an important protein source to complement grain proteins in Asian countries. Besides proteins, they contain various nutritious and functional components such as isoflavonoids, which help protect against metabolic diseases (*5*). Their fermentation makes new compounds derived from isoflavonoids, soy proteins, and dietary fibres, and glyceollins derived from isoflavonoids are also accumulated (*6*). Glyceollins (**Figure 1**) are phytoalexins converted from daidzein in soybeans with fungi infection, and they often act as antifungal, antibacterial, or anticancer compounds (*7*, *8*). As compared with the well-studied phytoalexin resveratrol found in fungus-infected grapevines and the skin of red grapes, less is known about the biological functions of glyceollins, but it is possible that these compounds have positive health effects on metabolic diseases such as obesity, inflammation, and cardiovascular diseases (*6*). Recent studies have found that glyceollins are candidate cancer preventative compounds for hormone-dependent tumors (*9*). Glyceollins have also been reported to mediate antihormonal effects through estrogen receptor-α and -β in MCF-7 cells, although they have greater antagonism toward estrogen receptor-α than estrogen receptor-β (*10*). Glyceollins are known to suppress the proliferation of breast cancer cells in postmenopausal female monkeys through their antiestrogenic action when estrogen activated proliferation (*11*). However, many studies have demonstrated that estrogen has antiobesity and antidiabetic effects and that postmenopausal women and ovariectomized animals exhibit the dysregulation of energy and glucose homeostasis (*12*, *13*). Thus, antiestrogenic compounds can disturb such homeostasis.

However, because of the recent controversies regarding the health risks/benefits of hormone replacement therapy in postmenopausal women (*14*), the metabolic properties of selective

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estrogen receptor modulators (SERMs) have been under evaluation. SERMs have estrogen receptor antagonists in breast and endometrial cells but exhibit estrogen receptor agonists in other tissues such as adipose and skeletal muscles (*15*, *16*). EM-652, a pure antiestrogen in human breast and uterine cancer cells, is an effective agent for preventing diet- and ovariectomy-induced obesity (*16*). Thus, it is of interest to study the effect of glyceollins, which are known to be antiestrogenic, on the parameters involved in glucose metabolism, such as insulin-stimulated glucose uptake, PPAR-γ agonistic action, and triacylglycerol accumulation in adipocytes, GSIS, β-cell apoptosis, and gene expression related to endoplasmic reticulum (ER) stress in insulinoma cells and glucagon-like peptide-1 (GLP-1) secretion in enteroendocrine cells in vitro.

MATERIALS AND METHODS

Fermentation of Soybeans. Soybeans (Tae-Kwang variety, 2008) were washed with 80% ethanol for 3 min and then rinsed with sterile deionized water three times to prevent contamination. They were soaked in autoclaved deionized water overnight, and the hull of the soybean was removed by hand. Halved soybeans were placed onto a filter paper (Whatman, Piscataway, NJ) in a 150 mm glass Petri dish and inoculated with spores resuspended in water (15 mL fungal suspension/200 g soaked soybeans). The dish was covered with parafilm and stored at 25 °C for 4 days in the dark until black spots began to form on the soybeans. Fungal spores were prepared as follows: *Rhizopus microsporus* var. *oligosporus* (ATCC 22959) was inoculated at the center of the potato dextrose agar (Difco, Franklin Lakes, NJ) and grown at 25 °C until mycelia covered the whole plate. Mycelia with spores were recovered using a sterile wood stick after a small volume of sterile water (1−2 mL) was added. After it was filtered with a sterile cheese cloth, the spore suspension was recovered. The number of spores was counted using a hemacytometer (Marienfeld, Germany), and the spore concentration was adjusted to 1×10^8/mL.

Preparation and Isolation of Glyceollins. Unfermented and fermented soybeans were extracted with methanol, and glyceollins were detected in the extract using high-performance liquid chromatography (HPLC) (Waters Co., Milford, MA). Separations by HPLC were carried out using a Gemini C18 (150 mm × 2.0 mm; 5 μm; Phenomenex, Torrance, CA) reversed phase column, with monitoring at a wavelength of 280 nm. Elution was carried out at a flow rate of 0.8 mL/min with the following solvent system: A = 0.1% phosphoric acid/water; B = acetonitrile; 10−35% B in 40 min, then 35 to 10% B for 5 min (in 45 min) followed by holding at 10% B for 10 min (in 55 min). Chromatograms definitely revealed that glyceollins existed only in fermented soybeans (**Figure 2**). Retention times for the isoflavonoids were as follows: daidzin, 6.7 min; genistin, 10.9 min; malonyldaidzin, 14.0 min; daidzein, 17.8 min; malonylgenistin, 18.4 min; genistein, 24.8 min; glyceollin III, 35.1 min; and glyceollin II/I, 36.0 min. We could resolve three peaks representing glyceollins I, II, and III, although glyceollin I/II were overlapping in our HPLC analysis condition. Although glyceollin III was separated by the HPLC condition, it was difficult to isolate for a further experiment. The groups of glyceollins were further resolved in a C18 column with acetonitrile and water at a flow rate of 1.0 mL/min, and their quantities were measured using an external standard of glyceollins generously provided by Dr. Stephen M. Boue at the Southern Regional Research Center, Agricultural Research Center, Louisiana State University (United States). The glyceollins were separated by high-performance thin-layer chromatography (TLC) for use in further experiments because the concentration was low and the amounts that we needed were small. A sample of the methanol extract was fractionated on aluminum-backed Silica gel 60 high-performance TLC plates (Merck, Damstadt, Germany) developed in chloroform:acetone:NH_4OH (5:5:0.1, v/v). The TLC-based fingerprint was produced using the CAMAG application system (Muttenz, Switzerland). Glyceollins were visualized with 20% aqueous H_2SO_4 spray reagent and UV light at 254 nm. A single glyceollin band (R_f = 0.5) was confirmed by the glyceollin standard and further purified by preparative TLC to give glyceollins. Glyceollins were dissolved in dimethyl sulfoxide (DMSO) for further experiments, and DMSO was used as a vehicle for the control.

Insulin-Stimulated Glucose Uptake in Vitro. The insulin-stimulated glucose uptake activity was analyzed by measuring the uptake of 2-deoxy-D-[^{3}H] glucose by 3T3-L1 adipocytes (*17*, *18*). Briefly, the adipocytes were seeded into 24-well plates at 4×10^4 cells per well in high glucose Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) containing fetal bovine serum (FBS) for 6 h (*17*). The medium was switched to low glucose DMEM containing 0.3% bovine serum albumin (BSA) and DMSO or 0.5 or 5 μM glyceollins and incubated at 37 °C for 16 h. The medium was switched to a Krebs−Ringer−Hepes (KRH) buffer containing either 0.2 or 10 nM insulin with or without DMSO or 0.5 or 5 μM glyceollins and further incubated at 37 °C for 30 min. At the end of the incubation, the glucose uptake was measured with 0.1 μCi 2-deoxy-D-[^{3}H] glucose and 1 mM glucose as the final concentrations for 10 min. The treatments with 10 nM insulin were used as a positive control. Nonspecific glucose uptake was measured in cells treated with DMSO or 0.5 or 5 μM glyceollins without insulin. The radioactivity retained by the cell lysates was determined using a Wallac Liquid Scintillation Counter (Waltham, MA).

Triacylglycerol Accumulation in 3T3-L1 Adipocytes. 3T3-L1 fibroblasts were grown and maintained and then differentiated into adipocytes as previously described by Shimaya et al. (*17*). To determine the effect of glyceollins on triacylglycerol accumulation, DMSO or 0.5 or 5 μM glyceollins was added into the medium with 0.2 nM insulin (Sigma Co., St. Louis, MO), 50 μM dexamethasone (Sigma Co.), and 0.8 mM isobutylmethyl xanthine (Sigma Co.) for 4 days during the differentiation of 3T3-L1 fibroblasts, and then, the cells were treated with DMSO or 0.5 or 5 μM glyceollins without differentiation inducers for 6 or more days. Until the cells were harvested, fresh DMSO or 0.5 or 5 μM glyceollins were added whenever the media were replaced. At the end of the 6 day incubation period, the cells were harvested with a lysis buffer without glycerol, and the triacylglycerol contents in the cells were measured using a Trinder kit (Young Dong Pharmaceutical Co., Seoul, Korea).

PPAR-γ Agonist Activity. HEK 293 cells were seeded into 96-well plates at 1×10^4 cells per well 24 h before transfection. The cells were transiently transfected with a PPRE-luciferase construct (firefly pGL3-DR-1-luciferase; 0.12 μg DNA/well), pSV-SPORT-PPAR-γ expression vector (0.12 μg DNA/well), and pSV-SPORT-retinoid X receptor (RXR)-α vector (0.08 μg DNA/well) with a lipofectamine plus reagent (Invitrogen) according to the manufacturer's protocol (*18*−*20*). These vectors were generously provided by Dr. Bruce Spiegelman (Department of Cell Biology, Harvard Medical School, United States). For an assessment of transfection efficiency, a renilla phRL-TK vector (10 ng DNA/well) was also transfected with a lipofectamine plus reagent. After 2 h of transfection, DMSO, 0.5 or 5 μM glyceollins, or rosiglitazone (Takeda Pharmaceuticals Inc., Lincolnshire, IL) was added into the medium for 40 h, and the medium was replaced by serum-free DMEM containing 0.1% BSA containing DMSO or 0.5 or 5 μM glyceollins for 12 h. The cells were washed in phosphate-buffered saline (PBS) three times and solubilized in a 1× passive lysis buffer (Promega, Madison, WI). Cell lysates were assayed for both firefly (PPRE-luciferase) and renilla luciferase activities using the Dual-Luciferase Reporter Assay System (Promega) and an Aureon PhL luminometer (Aureon Biosystems, Vienna, Austria). Ratios of firefly luciferase activity and renilla luciferase activity were calculated for the results.

GSIS and Cell Viability of Min6 Cells. Min6 cells between passages 19 and 30 were grown in high glucose DMEM containing 15% (v/v) heat-inactivated FBS, 50 μM β-mercaptoethanol, penicillin, and streptomycin at 37 °C in a humidified atmosphere with 5% CO_2. To induce the impairment of β-cell function and cell viability by fatty acid, palmitate/0.5% BSA conjugates were prepared as described previously (*21*). Briefly, 20 mM palmitate (Sigma) was solubilized by dropwise addition of 0.01 M NaOH at 70 °C for 30 min, and the palmitate soaps were then mixed with 5% fatty acid-free BSA in PBS at an 8:1 molar ratio of palmitate to BSA. The complex fatty acid was added to the serum-containing cell culture medium to obtain 500 μM palmitate. Min6 cells in a 24-well plate at 6×10^4 cells per well were incubated with high glucose DMEM containing 0.5% BSA or palmitate/0.5% BSA conjugate for 24 h to induce impairment. Those wells in 0.5% BSA or palmitate/0.5% BSA conjugate were divided into three groups, and each was assigned to DMSO or 0.5 or 5 μM glyceollins. Respective treatments lasted 24 h, and at the end of treatments, the Min6 cells of each well were washed with PBS, and the cells of each well

were treated with DMSO or 0.5 or 5 μM glyceollins in low glucose (2 mM) KRH buffers containing 20 mM Hepes, pH 7.4, and a palmitate/0.5% BSA conjugate as done previously. After 30 min of incubation, low glucose KRH buffer from the each well was taken, and the cells of each well were incubated in high glucose KRH solution for 30 min as done previously. At the end of the incubation, the high glucose KRH solution was separated. Afterward, these low and high KRH solutions were centrifuged at 3000 rpm for 30 min, and supernatants were collected. Exendin-4 (2.5 nM)-treated cells were used as a positive control. Insulin concentrations in supernatants from all cells were measured using a radioimmunoassay (RIA) kit (Linco Research, St. Charles, MO) and a Packard Cobra γ-counter (Packard Instrument Co., Inc., Meriden, CT).

Apoptotic cell death was measured in the Min6 cells treated with or without 500 μM palmitate and either DMSO, 0.5 or 5 μM glyceollins, or 2.5 nM exendin-4 with a cell death detection ELISA^{PLUS} from Roche Diagnostic Co. (Indianapolis, IN). This method detects the enrichment of mono- and oligonucleosomes in the cytoplasm of apoptotic cells, indicating apoptosis-associated DNA degradation. Cell death was quantified at 405 nm with an Aureon plate reader (Aureon Biosystems).

RNA Isolation and Reverse Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was isolated from Min6 cells treated with 500 μM palmitate and either DMSO, 0.5 or 5 μM glyceollins, or 2.5 nM exendin-4 for 24 h using Trizol reagent (Invitrogen), followed by extraction and precipitation with isopropyl alcohol. The cDNA was synthesized from equal amounts of total RNA with superscript III reverse transcriptase, and RT-PCR was performed with high fidelity Taq DNA polymerase. Equal amounts of cDNA were mixed with SyberGreen mix (BioRad, Richmond, CA) and specific primers for interested genes, and they were analyzed by a real-time PCR machine (BioRad). The mRNA levels of ER stress response genes, total X-box-binding protein-1 (XBP-1), activating transcription factor (ATF)-4, ATF-6, and C/EBP-homologous protein (CHOP) were determined. The expression level of the gene of interest was corrected for that of the housekeeping gene, glyceraldehyde 3-phosphate dehydrogenase (GAPDH) (*22*).

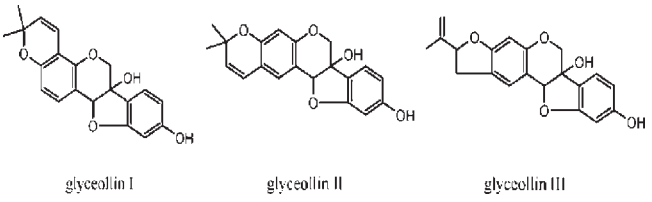

Figure 1. Chemical structures of glyceollins.

GLP-1 Secretion in NCI-H716 Cells. Human enteroendocrine NCI-H716 cells were maintained in suspension culture as described previously (*23*). Two days before the experiments, cells were seeded into 24-well culture plates precoated with Matrigel at 8×10^4 cells per well as described (*23*). On the day of the experiments, the supernatants were replaced by PBS containing 1 mM $CaCl_2$, 20 mM glucose, and a dipeptidyl peptidase IV inhibitor (pH 7.2). Cells were incubated for 8 h at 37 °C with DMSO or 0.5 or 5 μM glyceollins in RPMI containing 0.3% BSA and high glucose (20 mM). After the media were switched to a KRH solution with 20 mM glucose containing DMSO (control) or 0.5 or 5 μM glyceollins, GLP-1 concentrations from the supernatants were measured by RIA (Linco Research) and normalized by protein content.

Statistical Analysis. All results are expressed as a mean ± standard deviation (SD). Statistical analysis was performed using SAS version 9.1 (SAS Institute, Cary, NC). One-way analyses of variance (ANOVA) were carried out to determine the effect of high and low dosages of glyceollins. Multiple comparisons of the groups were undertaken by Tukey's tests. Comparisons between DMSO (control) and positive control were evaluated by two-sample t tests. $p < 0.05$ was considered significant.

RESULTS

Glyceollins in Fermented Soybeans. Fermented soybeans contained three isoforms of glyceollins (**Figure 1**). As shown in **Figure 2**, glyceollins existed in only fermented soybeans, not unfermented soybeans. Although glyceollin III had a separate peak, it was difficult to isolate, and a mixture of three glyceollins was used to investigate their antidiabetic actions. Because the proportion of glyceollin I, II, and III in the mixture was approximately 80, 10, and 10%, respectively, glyceollin I was the major component of the mixture. The amount detected by HPLC was 1.2 mg/g dry soybeans, based on the external standard of glyceollins.

Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes. Nonspecific glucose uptake (no treatment of insulin) occurred at a rate of 6.8 ± 1.5 dpm/μg protein and was not affected by treatment with glyceollins (no data shown). Insulin increased glucose uptake in a dose-dependent manner up to 10 nM, and the uptake reached a plateau (**Figure 3A**). Because insulin sensitizers increase

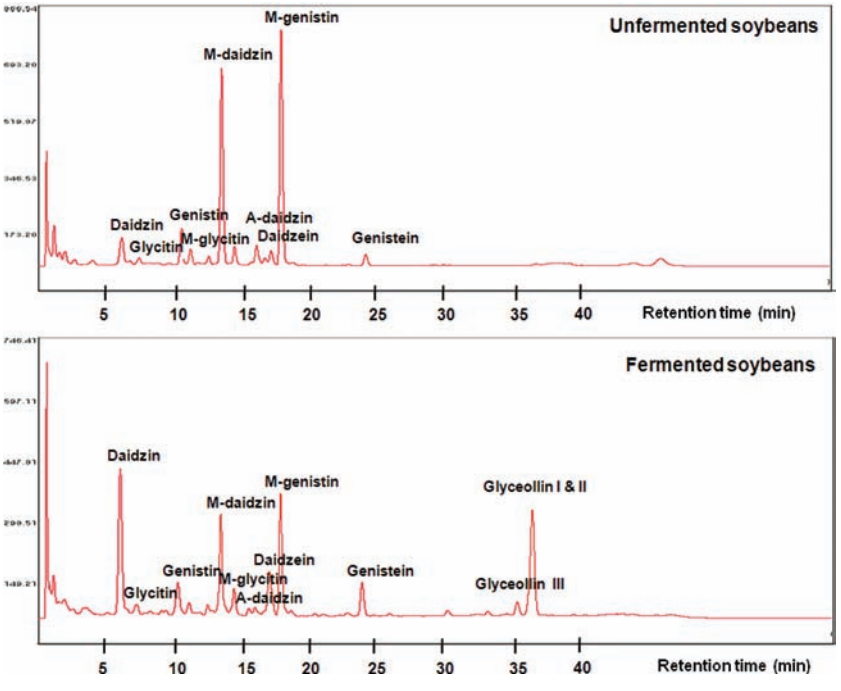

Figure 2. Chromatograms of the methanol extracts of unfermented soybeans and fermented ones with *R. microsporus* in HPLC analysis.

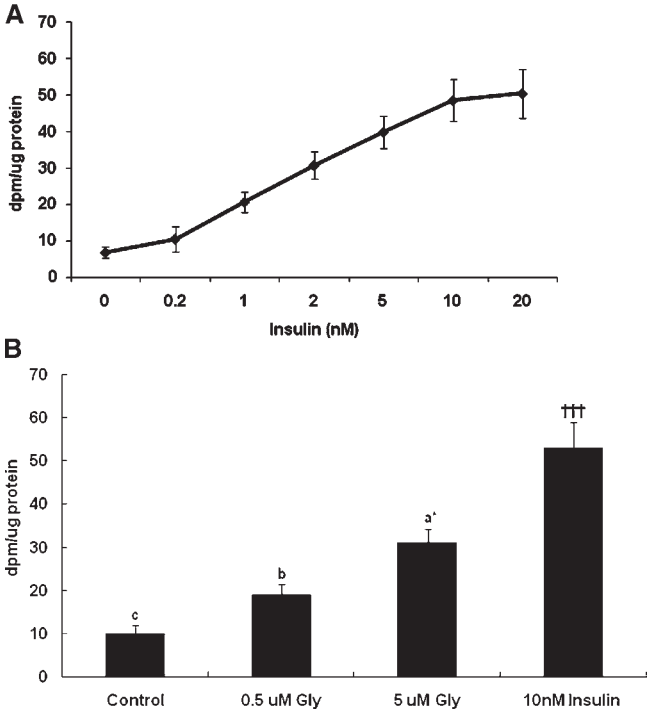

Figure 3. Insulin-stimulated glucose uptake in 3T3-L1 adipocytes. (**A**) Insulin-stimulated glucose uptake in 3T3-L1 adipocytes with the treatment of 0, 0.2, 1, 2, 5, 10, and 20 nM insulin. (**B**) Insulin-stimulated glucose uptake in 3T3-L1 adipocytes with the treatment of DMSO (control) or 0.5 or 5 μM glyceollins (Gly) plus 0.2 nM insulin. Rosiglitazone (2 μM) was used as a positive control. Each result is shown as the mean ± SD (n = 5). *Significant effect of glyceollins at $p < 0.05$. [a,b,c]Bars with different superscripts were significantly different at $p < 0.05$. †††Significant difference between the control and rosiglitazone at $p < 0.001$.

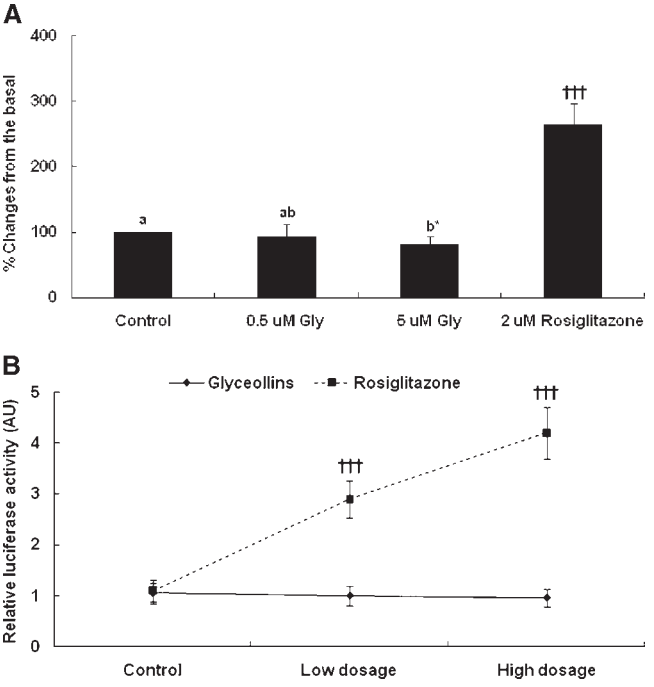

Figure 4. Triacylglycerol accumulation during differentiation from 3T3-L1 fibroblasts to adipocytes and PPAR-γ activity. (**A**) Triacylglycerol contents in 3T3-L1 adipocytes after incubating 3T3-L1 fibroblasts with the treatment of DMSO (control) or 0.5 or 5 μM glyceollins (Gly) plus differentiation inducers. Rosiglitazone (2 μM) was used as a positive control. (**B**) PPAR-γ activity in HEK 293 cells with treatment of DMSO (control) or 0.5 or 5 μM glyceollins (Gly) after transient transfection of a PPRE-luciferase construct, a pSV-SPORT-PPAR-γ expression vector, a pSV-SPORT-RXR-α vector, and a renilla phRL-TK vector. Each result is shown as the mean ± SD (n = 5). *Significant effect of glyceollins at $p < 0.05$. [a,b]Bars with different superscripts were significantly different at $p < 0.05$. †††Significant difference between the control and rosiglitazone at $p < 0.001$.

insulin-stimulated glucose uptake by enhancing insulin action at a low dose of insulin, cells were treated with 0.2 nM insulin plus DMSO (control) or glyceollins. Glyceollin treatment increased insulin-stimulated glucose uptake in a dose-dependent manner in comparison with the control. However, it did not increase glucose uptake as much as 10−20 nM insulin treatment (**Figure 3B**).

Differentiation and Triacylglycerol Accumulation in 3T3-L1 Adipocytes and PPAR-γ Agonist. The PPAR-γ agonist, known as an insulin sensitizer, increased triacylglycerol storage in adipose tissues with increasing insulin-stimulated glucose uptake (*24*). However, a high dose of glyceollins significantly decreased triacylglycerol accumulation in 3T3-L1 adipocytes in comparison with the control (**Figure 4A**). Rosiglitazone (2 μM), a commercial PPAR-γ agonist, elevated triacylglycerol storage by 264 ± 33% from the basal level (**Figure 4A**).

Glyceollins did not modulate PPAR-γ activity in HEK 293 cells transiently transfected with PPRE-luciferase, PPAR-γ, and RXR-α vectors in comparison with the control (**Figure 4B**). However, rosiglitazone, a PPAR-γ agonist, upregulated luciferase activity in a dose-dependent manner as compared with the DMSO treatment (**Figure 4B**). Thus, glyceollins did not work as PPAR-γ agonists.

GSIS and Cell Viability in Min6 Cells. In insulinoma Min6 cells, insulin secretion was 5.8 ± 0.7-fold higher in the high glucose (20 mM) DMEM medium than in the low glucose (2 mM) medium. Glyceollins and exendin-4, known insulinotropic agents, did not stimulate GSIS in low glucose media with or without treatment with palmitate, a long-chain saturated free fatty acid (**Figure 5A**). Glyceollins stimulated GSIS in the high glucose medium without the addition of 500 μM palmitate, but the stimulation was less potent than that by exendin-4 (2.5 nM) in the high glucose medium without palmitate (**Figure 5A**). Palmitate suppressed GSIS by over 3-fold in the high glucose medium, and glyceollins inhibited the suppression of GSIS with palmitate in a dose-dependent manner in the high glucose medium (**Figure 5B**). Exendin-4 strongly potentiated GSIS in the high glucose medium with palmitate (**Figure 5B**).

In addition, cell apoptosis decreased in the media without free fatty acids in response to glyceollins in a dose-dependent manner, but the decrease was not significant (P = 0.08). However, exendin-4 significantly reduced apoptotic cell death in the media without palmitate as compared with the control. Palmitate markedly increased apoptotic cell death in Min6 cells. Glyceollins decreased cell death in the media treated with palmitate in a dose-dependent manner, whereas exendin-4 greatly reduced cell death in comparison with the control (**Figure 5C**).

ER Stress in Min6 Cells. Because GSIS and β-cell death were induced with palmitate treatment in Min6 cells, the gene expression related to ER stress was measured. The incubation of Min6 cells with palmitate showed that a 500 μM palmitate treatment for 24 h, the dose-eliciting GSIS inhibition and reduction of cell viability, induced the expression of genes responding to ER stress, including XBP-1, ATF-4, ATF-6, and CHOP, as quantitatively measured by a real-time PCR (**Figure 6**). Consistent with GSIS and cell apoptosis, glyceollins decreased the expression of ER stress-related genes (XBP-1, ATF-4, ATF-6, and CHOP) in a dose-dependent manner in comparison with the control.

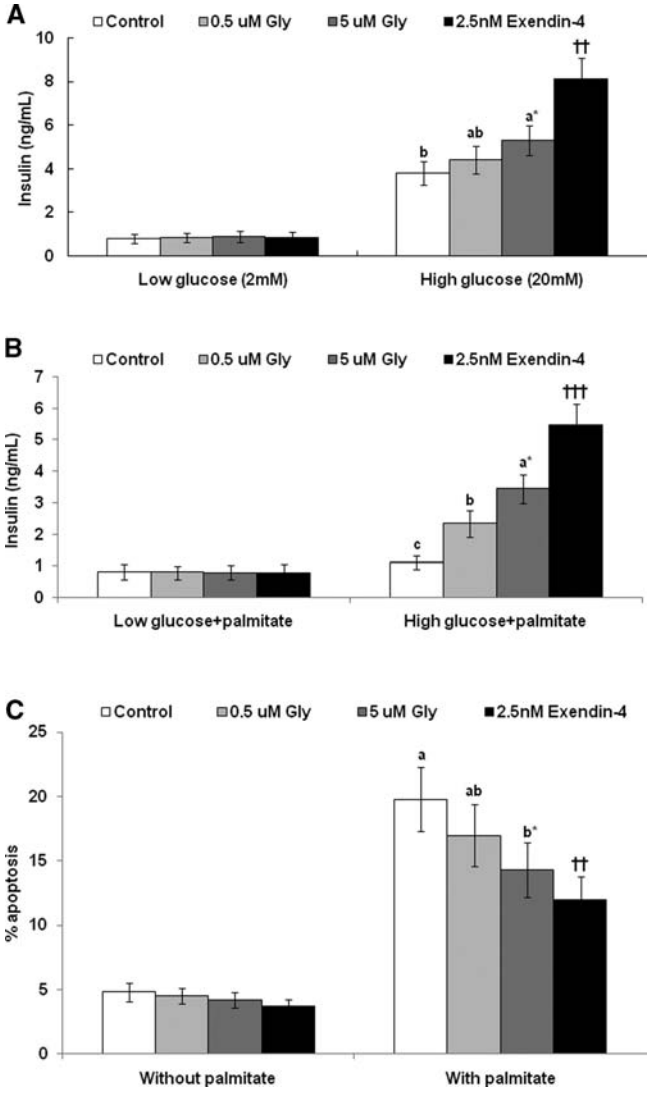

Figure 5. GSIS and cell apoptosis with and without treatment with palmitate in Min6 cells. (**A**) Insulin concentrations in the supernatant of Min6 cells incubated with the treatment of DMSO (control) and 0.5 or 5 μM glyceollins (Gly) without palmitate treatment. Exendin-4 (2.5 nM) was used as a positive control. (**B**) Insulin concentrations in the supernatant of Min6 cells incubated with the treatment of DMSO (control) or 0.5 or 5 μM glyceollins (Gly) with 500 μM palmitate treatment. (**C**) Cell apoptosis in Min6 cells with the treatment of DMSO (control) and 0.5 or 5 μM glyceollins (Gly) with and without 500 μM palmitate treatment in high glucose media (20 mM). Each result is shown as the mean $\pm$ SD (n = 5). *Significant effect of glyceollins at $p < 0.05$. [a,b,c]Bars with different superscripts were significantly different at $p < 0.05$. [††]Significant difference between the control and exendin-4 at $p < 0.001$. [†††]At $p < 0.001$.

Exendin-4 treatment markedly suppressed the induction of their expression in Min6 cells and was a better suppressor of gene expression related to ER stress than a high dosage of glyceollins. These findings suggest that palmitate caused ER stress in Min6 cells, which might play a role in the increase in β-cell apoptosis and inhibition of GSIS. Glyceollins worked as an ER stress reliever in Min6 cells.

GLP-1 Secretion in Enteroendocrine NCI-H716 Cells. Because GLP-1 secretion was enhanced with a high glucose solution in NCI-H716 cells (*23*), glyceollins were treated with 20 mM glucose to enhance GLP-1 secretion. Glyceollins increased GLP-1 secretion in a dose-dependent manner in enteroendocrine cells (**Figure 7**).

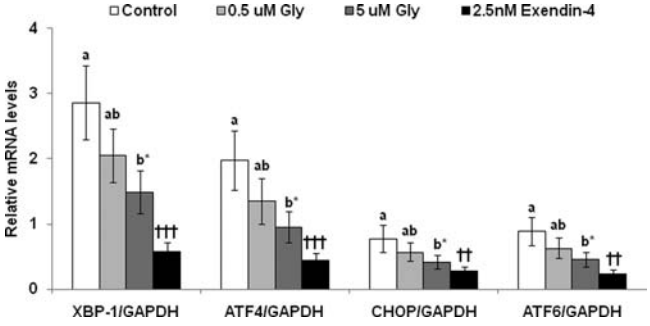

Figure 6. Expression of response genes to ER stress in Min6 cells. The mRNA levels of the ER stress response genes, XBP-1, ATF-4, ATF-6, and CHOP in Min6 cells with the treatment of DMSO (control) and 0.5 or 5 μM glyceollins (Gly). Exendin-4 (2.5 nM) was used as a positive control. Each result is shown as the mean $\pm$ SD (n = 5). *Significant effect of glyceollins at $p < 0.05$. [a,b]Bars with different superscripts were significantly different at $p < 0.05$. [††]Significant difference between the control and exendin-4 at $p < 0.01$. [†††]At $p < 0.001$.

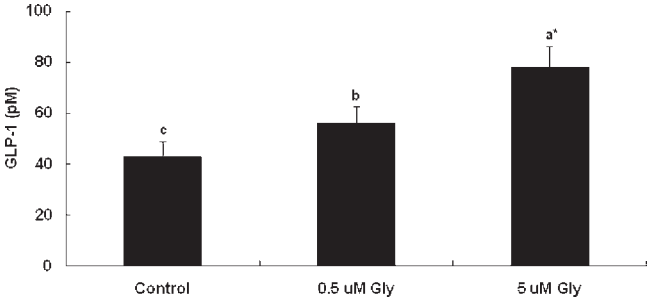

Figure 7. GLP-1 secretion in enteroendocrine NCI-H716 cells. The total GLP-1 content of the supernatants in enteroendocrine NCI-H716 cells incubated with DMSO (control) or 0.5 or 5 μM glyceollins (Gly) in high glucose media (20 mM). Each result is shown as the mean $\pm$ SD (n = 5). *Significant effect of glyceollins at $p < 0.05$. [a,b,c]Bars with different superscripts were significantly different at $p < 0.05$.

DISCUSSION

Phytoalexin glyceollins were derived from isoflavonoids under fungal stress. They had an antiestrogenic activity and a preventative action in hormone-dependent tumors in contrast to isoflavonoids. No other studies have investigated the effects of glyceollins on glucose metabolism. We examined their effects on glucose homeostasis and their mechanism in in vitro studies. Our results provide the new insight that glyceollins have an antiestrogenic action in breast and endometrial cells but can have estrogenic effects in other tissues in about their action. Glyceollins can belong to SERMs. They have a similar action to estrogen in adipocytes and insulinoma cells to enhance insulin-stimulated glucose uptake in 3T3-L1 adipocytes without increasing triglyceride accumulation. In addition, glyceollins potentiate GSIS in insulinoma cells and alleviate the palmitate-induced impairment of β-cell function and apoptosis via attenuating ER stress.

Although estrogen plays an important role in improving energy and glucose metabolism, it is used with caution in postmenopausal women because it increases the risk of breast and uterine cancer (*13*−*15*). In addition to estrogen itself, the effects of phytoestrogen on breast cancer have been studied (*25*), but the results are controversial. Recent studies have examined antiestrogenic agents such as tamoxifen and raloxifene because they have antiestrogenic effects on breast and endometrial cells but have estrogenic effects on other tissues (*16*). The compounds with properties of tamoxifen and raloxifene are SERMs, and they

represent a growing class of compounds that act as either estrogen receptor agonists or antagonists in a tissue-selective manner (*15*, *16*). This pharmacological profile might offer the opportunity to dissociate the favorable cardiovascular and diabetic effects of estrogen from the unfavorable hyperplasia effects on the breast and endometrium. Glyceollins are known antiestrogenic compounds, but they might work as estrogen agonists for islets and adipose tissues such as SERMs.

Although males were reported to have more lean mass and females to have higher adiposity, males were also found to have more visceral and hepatic adipose tissue, whereas females had more peripheral or subcutaneous adipose tissue (*26*). These differences, as well as differences in sex hormones and adipokines, can contribute to a more insulin-sensitive environment in females than in males despite the former being fatter. Lundholm et al. (*27*) reported that estrogen decreased the expression of genes involved in fatty acid synthesis, such as stearoyl-CoA desaturase, fatty acid synthase, acetyl-coenzyme A carboxylase α, fatty acid desaturase-1, and PPAR-γ in females. Changes in the expression of these genes were correlated with changes in plasma triacylglycerol levels. In addition, estrogen regulates fat accumulation in adipose tissues by the transcriptional control of lipoprotein lipase through the cAMP cascade and phosphoinositide cascade (*28*). Thus, a decrease in estrogen, as occurs with aging, tends to increase central obesity. These data seem to show that glyceollins decrease fat storage, possibly through an estrogen receptor agonist in the adipocytes, and also that glyceollins do not activate PPAR-γ in the adipose tissues. This suggests that glyceollins work as an estrogen receptor agonist in the adipocytes.

The insulinotropic effect of glyceollins has not yet been studied. The effects of genistein and daidzein on GSIS and β-cell proliferation are still controversial. However, genistein and daidzein seem to potentiate GSIS in a certain dose range through activation as consistent with estrogen-stimulated GSIS and β-cell proliferation (*13*, *29*, *30*). Genistein (0.1−5 μM) increased GSIS in insulinoma cells by augmenting cAMP accumulation to activate protein kinase A signaling in a dose-dependent manner in Min6 cells and islets (*29*). By contrast, there was no evidence that daidzein elevated GSIS in islets. In this study, glyceollins potentiated GSIS and prevented apoptosis in Min6 cells in a high glucose medium but not in a low glucose medium. This also suggests that glyceollins work as an estrogen agonist in insulinoma cells. To confirm the fact that glyceollins potentiated GSIS and prevented β-cell apoptosis in the situation of impaired β-cell function, glyceollins were simultaneously treated in insulinoma cells subjected to a long-term treatment (24 h) of palmitate. As shown in previous studies (*21*, *31*), a high dosage (500 μM) of palmitate induces the suppression of GSIS and an increase in β-cell apoptosis. Long-term treatment with glyceollins partly reverses palmitate-induced β-cell dysfunction and β-cell apoptosis in a dose-dependent manner, but the improvement was not as great as with exendin-4, a GLP-1 receptor agonist. The suppression of GSIS by palmitate was associated with reduced β-cell mass, which was the net result of β-cell proliferation and apoptosis. Kharroubi et al. (*31*) revealed that palmitate increased β-cell apoptosis partly by increased ER stress.

ER stress was suggested to be a mediator of free fatty acid-mediated β-cell death (*31*, *32*). ER stress comprises a series of cellular responses induced by several cues such as the overproduction of misfolding proteins, disorder in Ca^{2+} dynamics in the ER, oxidative stress, and exposure to cytokines (*32*). When ER stress increases, the production of chaperone proteins, translational attenuation, and degradation of the misfolding proteins ensues, eventually resulting in apoptosis (*31*, *32*). Other studies have shown that palmitate induced β-cell apoptosis and activated the protein kinase R-like ER kinase and inositol-requiring enzyme 1 pathways of the unfolded protein response (measured by ATF-4 and XBP-1 protein levels). Furthermore, palmitate upregulated the levels of the CHOP transcription factor, which in part mediates ER stress-induced cell death (*33*). As shown in a previous study, exendin-4 treatment partially blocked β-cell apoptosis with attenuating ER stress (*34*). Consistent with previous studies (*31*, *32*), the present study also exhibited the induction of ER stress by palmitate, whereas glyceollins reduced the ER stress, although not as much as exendin-4.

GLP-1 and its receptor agonist have been identified as new targets of insulinotropic action to alleviate diabetic symptoms. GLP-1 is an incretin (a peptide hormone) that is secreted from enteroendocrine L-cells and augments GSIS after the oral intake of glucose and free fatty acids (*35*). High glucose consumption elicits the release of GLP-1 secretion and GLP-1 stimulated GSIS from β-cells. Thus, substances that induce GLP-1 secretion or the GLP-1 receptor agonist can prevent and/or relieve diabetic symptoms. Exendin-4 has recently been approved for human use in type 2 diabetes because of its insulinotropic and weight-reducing properties. In the present study, glyceollins increased GLP-1 secretion in enteroendocrine NCI-H716 cells. Thus, increased circulating levels of GLP-1 by glyceollins can enhance the potentiation of GSIS and β-cell survival in vivo more than in vitro because they increase GLP-1 secretion, which might indirectly be responsible for stimulating GSIS and β-cell survival.

In summary, our observations support the hypothesis that glyceollins play a crucial role in glucose homeostasis by regulating glucose utilization in adipocytes and β-cell function and survival in insulinoma cells. The results provide new insight about their action as SERMs. Glyceollins have a similar action to estrogen in adipocytes and insulinoma cells, but they are known to show antiestrogenic activity in breast and endometrial cells. Glyceollins can be used as a novel therapeutic approach in glucose regulation after conducting experimental animal and human studies.

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