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Glyceollins, Soy Isoflavone Phytoalexins, Improve Oral Glucose Disposal by Stimulating Glucose Uptake

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ABSTRACT: Soy glyceollins, induced during stress, have been shown to inhibit cancer cell growth in vitro and in vivo. In the present study, we used prediabetic rats to examine the glyceollins effect on blood glucose. During an oral glucose tolerance test (OGTT), the blood glucose excursion was significantly decreased in the rats treated with oral administration of either 30 or 90 mg/kg glyceollins. Plasma analysis demonstrated that glyceollins are absorbed after oral administration, and duration of exposure extends from 20 min to at least 4 h postadministration. Exposure of 3T3-L1 adipocytes to glyceollins significantly increased both insulin-stimulated and basal glucose uptake. Basal glucose uptake was increased 1.5-fold by exposure to 5 μM glyceollin in a dose−response manner. Coincubation with insulin significantly stimulated maximal glucose uptake above basal uptake levels and tended to increase glucose uptake beyond the levels of either stimulus alone. On a molecular level, polymerase chain reaction showed significantly increased levels of glucose transporter GLUT4 mRNA in 3T3-L1 adipocytes, especially when the cells were exposed to 5 μM glyceollins for 3 h in vitro. It correlated with elevated protein levels of GLUT4 detected in the 5 μM glyceollin-treated cells. Thus, the simulative effect of the glyceollins on adipocyte glucose uptake was attributed to up-regulation of glucose transporters. These findings indicate potential benefits of the glyceollins as an intervention in prediabetic conditions as well as a treatment for type 1 and type 2 diabetes by increasing both the insulin-mediated and the basal, insulin-independent, glucose uptake by adipocytes.

KEYWORDS: *glyceollins, isoflavones, diabetes, glucose, adipocytes, glucose transporters*

INTRODUCTION

Over the last few decades, an abundance of processed foods in developed countries has resulted in a transition from diets high in carbohydrates and fiber to more animal-based diets with higher quantities of fats and sugars. However, data from a large European prospective study demonstrate that diets high in animal protein, but not diets rich in vegetable protein, are associated with an increased risk of type 2 diabetes (T2D).[1,2] Another prospective study suggests that a diet rich in soybeans and soy products may decrease the risk of becoming glucose intolerant, which may progress to T2D.[3] A recent large prospective study of the Japan Public Health Center indicates that obese postmenopausal women who consume the greatest levels of soy products are at significantly lower risk of developing T2D.[4]

The main bioactive molecules in soy are the isoflavones (e.g., genistein, daidzein, and glycitein). These occur in particularly high levels (5−20 mg/100 g) in traditional soy-based foods such as soybeans, soymilk, tofu, and foods with added soy flour or protein such as doughnuts, pancakes, and "power" type bars.[5] Isoflavones are studied in animal models and human volunteers as a mixture because soy isoflavone extracts are readily available. However, occasionally, specific compounds are isolated, purified, and studied. The administration of a mixture of the three standard isoflavones (genistein, daidzein, and glycitein) to obese Zucker rats improved their response to an oral glucose challenge, an effect proposed to be mediated by peroxisome proliferator activator receptor γ (PPAR γ).[6] Additional in vitro studies confirmed PPARγ activation by genistein and daidzein but not glycitein.[6] Data from another study confirmed PPARγ activation by daidzein and its metabolite, equol, which increases adipocyte differentiation and enhances glucose uptake.[7] However, other data implicate daidzein and genistein as inhibitors of glucose uptake in adipocytes.[8,9] Thus, an isoflavone mixture may not offer the best dietary supplement to manage glucose. Better efficacy may be realized by removing these constitutive isoflavones from the extracts. Although genistein has been shown to inhibit glucose uptake, derivatives of genistein were shown to increase both basal and insulin-stimulated glucose uptake in L6 myotubes.[10]

Some isoflavones are only induced by the plant's defense against pathogens such as bacteria and fungi in the soil; such compounds are termed phytoalexins. Three very similar

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phytoalexins called glyceollin I, glyceollin II, and glyceollin III are produced by soy when it is challenged by inoculating the young soy plant or seed with soil microorganisms, UV light, or heavy metals.[11] The glyceollins have been shown to block estrogen-mediated cell proliferation in vitro,[12] inhibit cell proliferation in mouse xenograft breast cancer models,[13] and regulate genes involved in lipid and carbohydrate metabolism in postmenopausal monkeys.[14] Recently, the glyceollins are reported to enhance insulin-mediated glucose uptake by 3T3-L1 adipocytes, increase insulin release from pancreatic β-cells, increase glucagon-like peptide-I (GLP-I) release from a human colon cell line, and prevent pancreatic β-cell apoptosis.[15] In a diabetic mouse model, administration of a fermented soybean diet containing the glyceollins as well as the isoflavone mixture mentioned above improved the mouse's blood glucose response to an oral glucose challenge when compared to diabetic mice that were only provided with an unfermented soy diet not containing glyceollins but containing other isoflavones.[16]

We were interested in testing whether the mixture of glyceollins would improve glucose regulation when administered alone to a prediabetic animal model to remove the possible interactions with other isoflavones. Furthermore, we wanted to be assured that the glyceollins are bioavailable after oral administration in the same model. We observed an improved tolerance to an oral glucose load if the animals were pretreated with glyceollins, and we report exposure of the experimental animals to the glyceollins for the duration of the tests by measuring blood levels of the glyceollins. We demonstrate that the enhanced glucose disposal may be a result of the glyceollins directly stimulating glucose uptake by adipocytes, a result of increased biosynthesis of the glucose transporter GLUT4.

MATERIALS AND METHODS

Glyceollins Preparation. A mixture of glyceollins I, II, and III was isolated using a procedure developed at the Southern Regional Research Center (Agricultural Research Service, U.S. Department of Agriculture, New Orleans, LA).[12,13] Soybean seeds (1 kg) were sliced and inoculated with *Aspergillus sojae*. After 3 days, the glyceollins were extracted from the inoculated seeds with 1 L of methanol (Sigma Chemical Co., St. Louis, MO). The glyceollins were isolated using preparative scale high-performance liquid chromatography (HPLC) using two Waters 25 cm 10 μm particle size μBondapak C_{18} radial compression column segments combined with an extension tube. HPLC was performed on a Waters 600E System Controller combined with a Waters UV−vis 996 detector. Elution was carried out at a flow rate of 8.0 mL/min with the following solvent system: A = acetonitrile (Sigma Chemical Co.), B = water (Millipore system); 5% A for 10 min, then 5−90% A in 60 min followed by holding at 90% A for 20 min. The injection volume was 20 mL. The fraction containing the glyceollins was concentrated under vacuum, lyophilized, and resuspended in dimethyl sulfoxide (DMSO) and Poloxamer 407 (Sigma Chemical Co.) for in vitro and in vivo studies, respectively. The glyceollins were confirmed by UV−vis spectrophotometry as a mixture of glyceollins I (68%), II (21%), and III (11%).

Prediabetic Rodent Model. Male ZDSD/Pco rats (PreClinOmics, Indianapolis, IN) were individually housed in suspended wire cages and maintained on a 12:12 h light:dark cycle under standard laboratory conditions with a controlled room temperature (20−21 °C). The protocol and all procedures were approved by the Institutional Animal Care and Use Committee of PreClinOmics, and the experiments were performed by the trained staff at PreClinOmics. Fifteen week old rats with a body weight of approximately 500 g were chosen for the experiments based on their prediabetic conditions. Diabetic synchronization can be achieved by feeding a caloric-dense diet. Rats received Purina 5008 chow (Ralston Purina, Belmont, CA) to maintain a prediabetic state. Chow was placed in hoppers for accurate food intake measurements, and experimental animals had free access to drinking water.

Glyceollins Exposure Study. Glyceollins were administered via oral gavage (3 mL). The study design included the following groups (n = 3 rats per group): vehicle (Poloxamer 407; 7.5% in water), glyceollins dissolved in Poloxamer to administer 30 and 90 mg/kg. Blood levels of glyceollins were measured 0.5, 1, 2, and 4 h after oral gavage. The animals were euthanized by decapitation, and trunk blood was collected into ethylenediaminetetraacetic acid (EDTA)-coated tubes supplemented with aprotinin. Plasma was separated and stored at −80 °C until analysis by HPLC−electrospray ionization (ESI)−MS/MS (see below).

Oral Glucose Tolerance Test (OGTT). Food was removed at the onset of the dark cycle, and the rats were fasted for 5 h.[17] Glyceollins were administered via oral gavage as described above except that there were eight rats in each group for this experiment. After 1 h, the rats were dosed with glucose (2 g/kg, 10 mL/kg, po). Tail vein blood was sampled for glucose measurement at 15, 30, 60, 90, and 120 min relative to the glucose challenge. Whole blood glucose levels were measured using an AlphaTrak blood glucose monitor (Abbott Laboratories, Abbott Park, IL). Data represent the mean ± standard error of the mean (SEM) of the blood glucose value for eight rats.

3T3-L1 Mouse Adipocyte Cell Culture. The method for cell culture was based on a previously reported method with some modifications.[18] Murine preadipocytes (Zen-Bio Inc., Research Triangle Park, NC) were cultured using PM-1-L1 medium (Zen-Bio Inc.) containing Dulbecco's modified Eagle's medium (DMEM)/Ham's F-10 medium (1:1, v/v), 15 mM *N*-2-hydroxyethylpiperazine-*N*′-2-ethanesulfonic acid (HEPES) (pH 7.4), 10% (v/v) of fetal bovine serum, penicillin (100 U/mL), streptomycin (100 mg/mL), and amphotericin B (0.25 μg/mL) in a humidified atmosphere (5% CO_2/95% air). After 3−4 days, when the cells reached confluency, the PM medium was replaced by the differentiation medium (DM-2-L1, Zen-Bio Inc.) containing DMEM/Ham's F-10 medium (1:1, v/v), 15 mM HEPES (pH 7.4), 3% (v/v) fetal bovine serum, biotin (33 μM), pantothenate (17 μM), human insulin (100 nM), dexamethasone (1 μM), penicillin (100 U/mL), streptomycin (100 μg/mL), amphotericin B (0.25 μg/mL), isobutylmethylxanthine (0.20 μM), and PPARγ agonist (10 μM) for 3 days. The medium was then changed to AM-1-L1 medium (Zen-Bio Inc.) containing DMEM/Ham's F-10 medium (1:1, v/v), 15 mM HEPES (pH 7.4), 3% (v/v) fetal bovine serum, biotin (33 μM), pantothenate (17 μM), human insulin (100 nM), dexamethasone (1 μM), penicillin (100 U/mL), streptomycin (100 μg/mL), and amphotericin B (0.25 μg/mL). The AM-1-L1 media were changed every 2−3 days during an additional 10 days of incubation.

Glucose Uptake Assay. The glucose uptake measurement was carried out according to the method described by Park et al. with some modifications.[15] Adipocytes were grown in 24-well plates and used 10−11 days after initiation of differentiation. Adipocytes were rinsed in sterile, fresh Krebs−Ringer−HEPES (KRH) buffer (HEPES pH = 7.4, 1 mM $CaCl_2$, 1.2 mM $MgSO_4$, 1 mM KH_2PO_4, 1.4 mM KCl, and 20 and 130 mM NaCl), and then preincubated for 24 h in KRH buffer. Then, the buffer was removed, and adipocytes were incubated in KRH buffer containing glyceollins for specified time period. Ten microliters of [^{3}H]-2-deoxy-D-glucose (Vitrax, Placentia, CA) diluted to 0.01 μCi/μL with D-glucose (100 mM) was added to each well and incubated for 10 min in a 37 °C water bath. The supernatant was removed, and plates were rinsed rapidly three times with ice cold KRH. The final rinse was aspirated, being careful to not remove the cellular monolayer, and 500 μL of ice cold RIPA buffer (Sigma Chemical Co.) was added to lyse the cells. The cellular content in each well was triturated with a 1 mL pipet several times to remove attached cells and cellular components from the bottom of the plate. A 450 μL aliquot was added to 5 mL of Ecolume scintillation fluid (MP Biomedical, Santa Ana, CA). The vials were mixed and counted for 10 min in an Applied Biosystems 1100 liquid scintillation counter using the factory preset window to detect tritium. Data are the average of three experiments

that were normalized by calculating the percent cpm glucose uptake as compared to basal cpm glucose uptake.

RNA Extraction and Quantitative RT-Polymerase Chain Reaction (PCR). RNA isolation and RT-PCR were carried out according to previously reported methods.[18] Adipocytes were grown in six-well plates and used at days 10−11 after initiation of differentiation. Adipocytes were rinsed in sterile KRH buffer and then preincubated for 24 h in KRH buffer. The buffer was removed, and adipocytes were treated with either DMSO as a vehicle or glyceollins for 3 h. The total RNA was isolated using Trizol reagent (Invitrogen) and purified on RNeasy columns (Qiagen, Valencia, CA) according to the manufacturer's protocol. The RNA quality and concentration were determined by absorbance at 260 and 280 nm. The total RNA was reverse-transcribed using the QuantiTect Reverse Transcription kit (Qiagen) according to the manufacturer instructions. The sequences of the forward primer, reverse primer, and TaqMan probes for GLUT1, GLUT4, and the housekeeping gene ribosomal protein L32 (RPL32) (NM_172086) were described previously.[18] The PCR reactions were performed in 96-well plates in a CFX96 Real-Time PCR Detection Systems (Bio-Rad, Hercules, CA). The thermal cycle conditions were as follows: 2 min at 50 °C and 10 min at 95 °C, followed by 50 cycles at 95 °C for 15 s each, and 60 °C for 60 s. The $\Delta\Delta C_T$ method of relative quantification was used to determine the fold change in the gene expression levels in the glyceollin-treated adipocytes as compared to the DMSO-treated control.[19] The resulting threshold cycle (C_T) values of the target mRNAs were normalized to the C_T values of the internal control Rpl32 in all samples.

Western Blots. Western blots were carried out according to previously reported methods with some modifications.[18] 3T3 L1 mouse preadipocytes were differentiated to the adipocyte stage in 10 cm culture dishes, incubated in the KRB buffer for 24 h, and treated with DMSO as a vehicle or glyceollin mix at a concentration of 0.1, 1.0, or 5 μM for 3 h. After the treatment, the cells were harvested with 0.5 mM EDTA in phosphate-buffered saline (PBS), and total proteins were isolated using M-Per mammalian protein extraction reagent (Thermo Scientific) supplemented with proteinase inhibitors (Sigma). The protein samples were separated via polyacrylamide gel electrophoresis (PAGE), transferred to the nitrocellulose membrane (Invitrogen), blocked with 5% skim milk, and then incubated with primary antibodies specific to GLUT1 (Abcam), GLUT4 (Abcam), β-actin (Cell signaling), and IRDye-conjugated secondary antibodies. The resulting bands were visualized and quantified using an Infrared Image Analyzer (Li-COR Bioscience).

HPLC-ESI-MS/MS Analysis of Glyceollins in Plasma. Frozen (−80 °C) plasma samples were thawed, and 120 μL was transferred into a 1500 μL microcentrifuge tube. An extraction was performed by adding 500 μL of ethyl acetate to the tube, vortexing, and then centrifuging for 5 min at 16100*g*. A 450 μL aliquot of the supernatant was carefully withdrawn, placed in a 500 μL microcentrifuge tube, and vacuum centrifuged at room temperature until dry. The precipitate was resuspended in 220 μL of methanol, centrifuged again for 5 min at 16100*g*, transferred to autosampler vials, and stored at 4 °C until injection. Plasma standards were made by spiking blank rat plasma with 5−10000 ng/mL (0.015−29.6 μM) of glyceollin.[20] Fresh plasma standards were prepared, processed, and analyzed with each set of samples, generating a new calibration curve for each run.

Chromatographic separation was performed using isocratic elution at ambient temperature. The Waters HPLC system (Waters Corp., Milford, MA) consisted of a model 2695 separations module, equipped with quaternary pump, a degasser, thermostatted column, and autosampler. The analytical HPLC column was an Ascentis Express, C18 column (7.5 mm × 2.1 mm, 2.7 μm), protected by a Supelguard Ascentis C18 guard column (2 cm × 2.1 mm, 3 μm), both from Supelco (Sigma-Aldrich). The sample injection volume was 10 μL, with separation achieved using an isocratic composition of solvent A (0.1% formic acid in water) and solvent B (0.1% formic acid in methanol) in a ratio of 20:80 at a flow rate of 0.2 mL/min. The eluent was introduced directly into the electrospray source of a Quattro Micromass triple quadrupole mass spectrometer that was set in the positive ionization mode. Optimization of instrument response was performed by direct infusion of synthesized standard glyceollin in the mass spectrometer. The electrospray source was operated using nitrogen at flow rates of 50 (nebulizer gas) and 750 L/h (desolvation gas). The ion source and desolvation temperatures were set at 100 and 400 °C, respectively. The capillary voltage was 3.5 kV, and the cone voltage was 45 V. Glyceollin ions were fragmented at a collision energy of 32 eV using argon as the collision gas. Multiple reaction monitoring (MRM) mode was used for quantification of total glyceollin (glyceollin I, II, and III) levels by monitoring the ion transition m/z 321.05 → 305.11. The parent ion in this transition represents glyceollin after it has lost a water moiety during the ionization process. Mass-Lynx version 4.0 software was employed for instrument control, data acquisition, and analysis.

Statistical Analysis. All results are expressed as means ± SEMs. Plasma glyceollin values were measured in triplicate, and that mean value was used to summarize the data observed from all animals at each time point. Plasma glucose levels during the OGTT were measured in duplicate, and those mean levels from all animals at each time point were summarized. 3T3-L1 studies were performed with multiple passages of the cell line. Each experiment was performed in triplicate wells. Glucose uptake values were averaged, and the mean value was normalized to the mean value for basal glucose uptake. Each dose−response study was contained in a single 24-well plate, which was repeated multiple times, and the data were pooled. A four-parameter logistic equation was used to compute the EC_{50}. Statistical comparisons for each experiment were performed using one-way analysis of variance (ANOVA) or two-way ANOVA with Dunnett's multiple comparison post-test. A p value below 0.05 was considered statistically significant. All statistical analyses were performed by using SigmaPlot for Windows version 11.0 (Systat Software, Inc.).

RESULTS

Exposure to Glyceollins after Oral Gavage. To determine if adipocytes were exposed to glyceollins after oral gavage, plasma levels of the phytoalexins were measured for 4 h following glyceollin administration. Glyceollins were quantitated in all plasma samples (Figure 1). Total glyceollin levels appeared in the plasma by 20 min, and they remained stable during the next 3 h and 40 min. Glyceollin levels ranged from 0.24 to 0.35 μM (81.2−118.4 ng/mL) for the 30 mg/kg dose and from 0.35 to 0.47 μM (118.2−159.0 ng/mL) for the 90

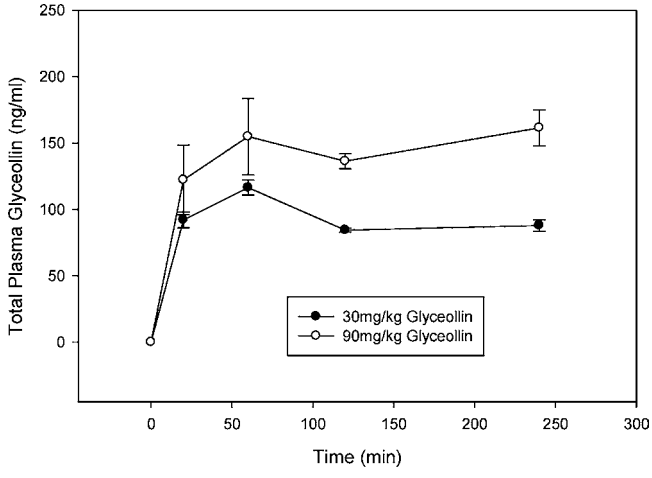

Figure 1. Plasma levels of glyceollins in ZDSD/Pco rats after administration of glyceollins (30 and 90 mg/kg, po). Values represent the mean ± SEM from three different rats at each time point and dose. Plasma concentrations of glyceollin I, glyceollin II, and glyceollin III, measured by MS/MS as described in the Materials and Methods, were combined and summed to calculate the total plasma glyceollin (ng/mL).

mg/kg dose. These data demonstrate that glyceollins are absorbed after oral administration, and the duration of exposure extends from 20 min to at least 4 h postadministration.

OGTT. The ZDSD/Pco rats were in a prediabetic state as evidenced by the fasting blood glucose value of 127.6 ± 1.5 mg/dL (n = 24). Blood glucose increased to a maximum level at 30 min after the oral glucose gavage, and it remained elevated in the vehicle group until 60 min but was significantly (p < 0.05) less at that time in both glyceollin groups (Figure 2).

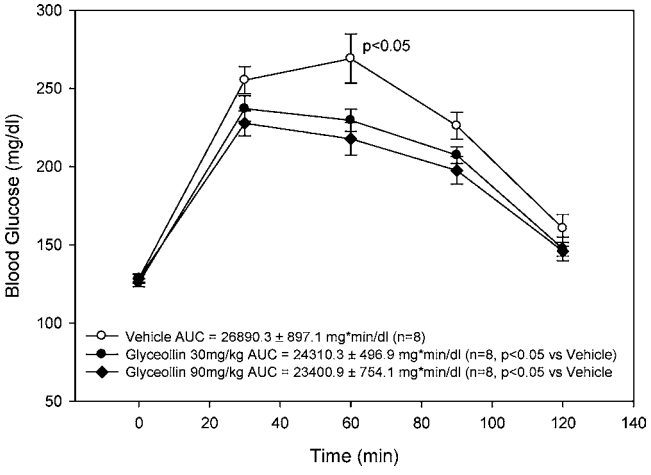

Figure 2. Blood glucose levels of prediabetic ZDSD/Pco rats after administration of glucose (2 g/kg, po; time 0). Glyceollins were administered (po) 1 h prior to the OGTT. At 60 min, the blood glucose levels for the glyceollin-treated animals were significantly less than the vehicle-treated rats, and the area under the curves for the glyceollin groups was significantly less than that integrated for the vehicle-treated rats.

Disposal of the circulating glucose during the 120 min period of the oral challenge was significantly (p < 0.05) greater in both glyceollin groups than in the vehicle group: the area under the blood glucose curve (AUC) for the OGTT was 26890 ± 876, 24310 ± 496, and 23401 ± 754 mg min/dL, for the vehicle group (n = 8), 30 mg/kg glyceollin group (n = 8), and 90 mg/kg glyceollin group (n = 8), respectively. There was no significant difference between the two glyceollin groups.

Glyceollins Stimulate Both Basal- and Insulin-Mediated Glucose Uptake in 3T3-L1 Adipocytes. To determine whether the underlying mechanism of the glyceollin-mediated improvement of the oral glucose challenge in a prediabetic rat model involved increasing the glucose uptake by fat, we studied glyceollin pharmacology in 3T3-L1 cells. These murine cells were obtained as preadipocytes, differentiated into mature adipocytes, and exposed to 0.3−300 nM insulin. Glucose uptake by the 3T3-L1 adipocytes increased in response to the insulin in a dose-dependent manner (Figure 3). Maximal stimulation was more than 3-fold of that measured for basal glucose uptake, and the concentration of insulin that produced half of that response (EC_{50}) was calculated to be 1.92 ± 1.54 nM (n = 3).

To measure if glyceollins potentiate the adipocyte's response to insulin, 3T3-L1 differentiated cells were exposed to either insulin (0.3 nM) alone or glyceollins (5 μM) alone and both in combination for 3 h (Figure 4). Glucose uptake was greatest when the cells were coincubated with 5 μM glyceollins and insulin. That stimulation was significantly (p < 0.05) greater

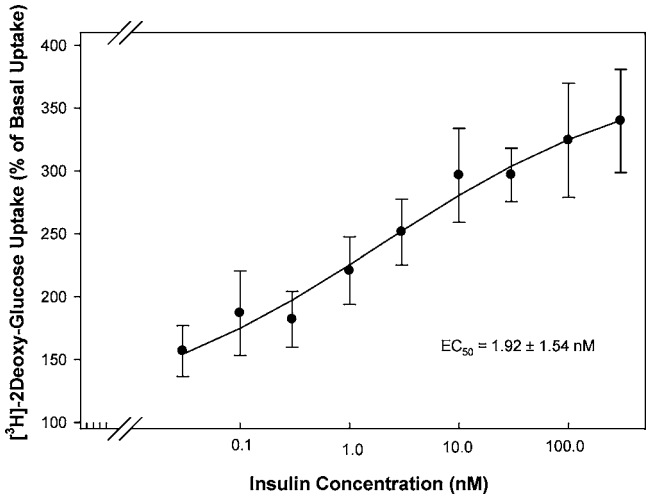

Figure 3. Insulin-mediated glucose uptake by 3T3-L1 adipocytes. Cells were exposed to insulin for 30 min at 37 °C followed by 10 min of incubation with [^{3}H]-2-deoxy-glucose. The effective concentration for 50% increase in glucose uptake (EC_{50}) was 1.92 nM. The symbols represent means ± SEMs.

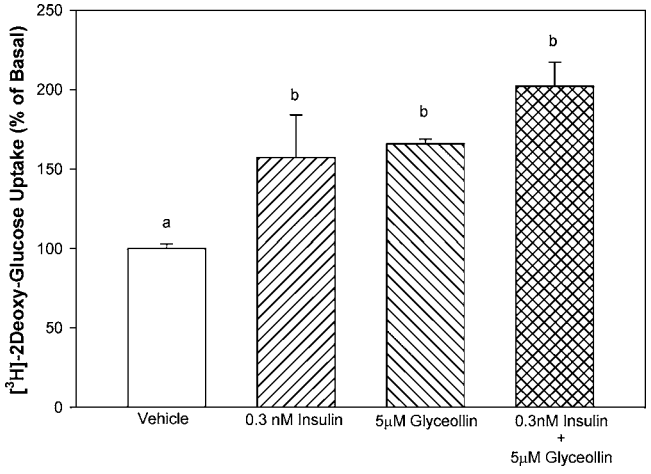

Figure 4. Glyceollins, insulin, and glyceollins combined with insulin-stimulated glucose uptake by 3T3-L1 adipocytes. Adipocytes were exposed to inulin, glyceollins, or both for 3 h. The symbols represent means ± SEMs. All means for insulin-stimulated glucose uptake with different letters are significantly (p < 0.05) different.

than basal glucose uptake and tended to be greater than the responses to either glyceollins or insulin alone.

Additional experiments using different exposure times for the glyceollin pretreatment demonstrated that a 45 min time period of glyceollin exposure was sufficient for enhancing insulin-stimulated glucose uptake (data not shown). To further investigate the stimulative glyceollin effect on glucose uptake by adipocytes, we performed glyceollin dose−response studies (Figure 5). Glucose uptake was stimulated by 45 min of exposure to glyceollins at doses ranging between 0.5 and 10 μM with an EC_{50} of 2.40 ± 0.43 μM and a maximal uptake of 2.04 ± 0.24-fold stimulation above basal glucose uptake (n = 3).

Effect of Glyceollin on GLUT1 and GLUT4 mRNA. To determine the underlying mechanism for glyceollin stimulation of glucose uptake, we examined the expression of the genes encoding GLUT1 and GLUT4, which are the key GLUT genes expressed in adipocytes. Real-time PCR assay revealed

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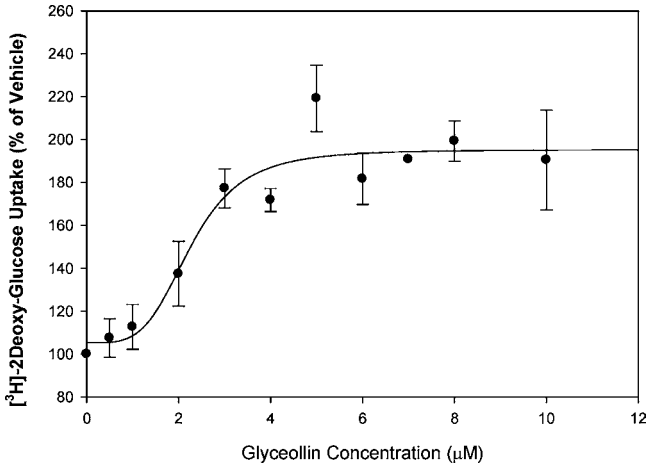

Figure 5. Glyceollin-mediated glucose uptake by 3T3-L1 adipocytes. Cells were exposed to glyceollin for 45 min at 37 °C followed by 10 min of incubation with [^{3}H]-2-deoxy-glucose. The EC_{50} was 2.40 ± 0.43 μM and a maximal uptake of 2.04 ± 0.24-fold (computed by the 4-parameter logistic equation). The symbols represent means ± SEMs.

significantly ($p < 0.05$) increased expression of both GLUT1 and GLUT4 in cells exposed to 1−5 μM glyceollin as compared to the DMSO (vehicle)-treated cells (Figure 6).

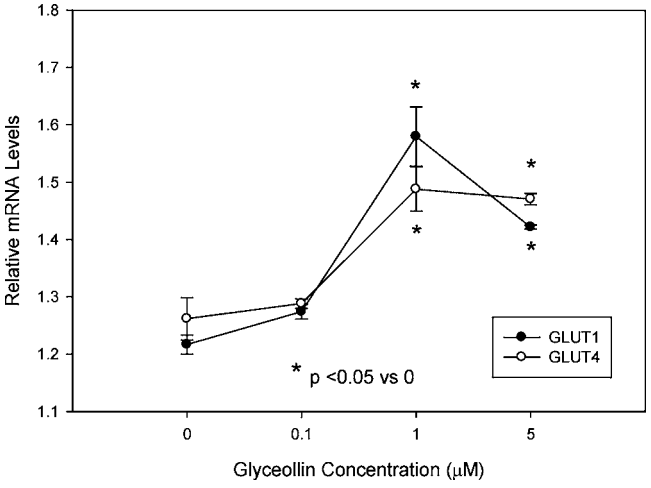

Figure 6. Glyceollins stimulated the expression of GLUT genes GLUT1 and GLUT4 in 3T3-L1 adipocytes. mRNA levels of both genes were measured by real-time PCR and are shown relative to mRNA level of RPL32. The cells were exposed to glyceollin for 3 h, mRNA was isolated from the cells, cDNA was synthesized, and gene expression was quantitated by real-time PCR. Symbols represent means ± SEMs.

Effect of Glyceollin on GLUT1 and GLUT4 Protein Levels. Increased levels of GLUTs GLUT1 and GLUT4 were observed in adipocytes treated with 5 μM glyceollins for 3 h. When the protein quantities detected via Western blotting were normalized to β-actin protein levels, there was a significant 1.7-fold increase in GLUT4 protein levels in 5 μM glyceollin-treated cells in comparison to the low-dose glyceollin-treated cells and the untreated control. There was also a 1.4-fold increase in the GLUT1 protein level (Figure 7). The latter was not significant due to a greater variability within the treatment groups.

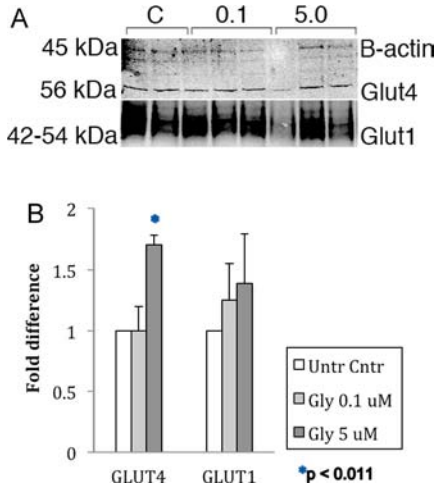

Figure 7. Glyceollins increased GLUT1 and GLUT4 protein levels. Western blotting of total proteins isolated from mouse adipocytes treated with 0.1 and 5.0 μM glyceollins (lines 0.1 and 5.0) or untreated control cells (lines "C"). The proteins were separated via PAGE, transferred on the nitrocellulose membrane, and incubated with antibodies specific to the GLUTs 1 and 4 (Glut1 and Glut4, respectively) and β-actin (A). For quantification purposes, the levels of the proteins were normalized to the levels of the β-actin (B). Protein levels of both GLUT 1 and GLUT4 increased in the adipocytes treated with 5.0 μM glyceollins 1.4- and 1.7-fold, respectively. Importantly, the increased GLUT 4 level differed from all other treatment groups (α = 0.011).

DISCUSSION

Recently, soybean and soy-derived products have become increasingly popular in Western diets due to their health-promoting claims, although the bioactive molecules responsible for such effects remain unknown. Among candidates are soy isoflavones genistein, daidzein, and more recently the glyceollins, all of which were shown to participate in glucose homeostasis. Genistein and daidzein have been studied intensively; however, the published data are controversial. Daidzein increased insulin-stimulated glucose uptake in a dose-dependent manner by increasing the abundance of insulin-responsive GLUT4 in vitro.[7] Furthermore, Meezan et al.[21] demonstrated that daidzin (75 mg/kg) administration (ip and po) to obese mice increased blood glucose levels during an OGTT. Other studies offer evidence that both daidzein and genistein inhibit insulin-induced glucose uptake in adipocytes.[8,9] Glyceollins, in contrast, are shown to improve glucose metabolism at the cellular level,[15] although the mechanism is not clear.

The glyceollins are daidzein derivates with a modified pterocarpan ring structure.[12,13,22] Intermediate structures in the biosynthesis of glyceollins from daidzein in soy are glycinol[23−25] and glyceollidins I and II.[22,24,25] Glycinol and glyceollidins I and II have been listed as components of soy challenged by *Rhizopus microporus* var. *oryzae*.[24,25] We observed that glycinol could not stimulate glucose uptake in 3T3-L1 adipocytes (data not shown), demonstrating the selectivity for glyceollin over very closely related isoflavones. Besides increasing pterocarpan isoflavones, soy fermentation increases the concentration of glycosylated forms of daidzein that can be converted to aglycones in the proximal intestine.[26] An approximate 5−6-fold increase in daidzin concentrations was determined in fermented soy when compared to unfermented

soy.[16] This increased daidzin can be converted to the aglycone that has the ability to alter insulin-mediated glucose uptake.[7,8]

In our study, we showed that blood levels of glyceollins after oral administration were related to lower blood glucose levels of prediabetic rats. Our results demonstrate that a 1 h pretreatment with a mixture of three glyceollins improved the blood glucose response of prediabetic ZDSD/Pco rats to an oral glucose challenge. The improved glucose disposal in prediabetic rats may be associated with the increased glucose uptake by adipocytes. In this work, we demonstrated that glyceollins not only increase insulin-mediated glucose uptake by fat cells, but more importantly, glyceollins stimulate glucose uptake independent of insulin. Although a stimulative effect of glyceollins on the insulin-mediated glucose uptake by 3T3-L1 adipocytes has been documented,[15] there was no effect of glyceollins on insulin-independent glucose uptake in 3T3-L1 cells observed in that study possibly due to the extended exposure of the adipocytes to glyceollin—about 16 h[15] as compared to 45 min and 3 h of exposure used in our study. Furthermore, our data indicate a dose-dependent up-regulation of GLUT4 gene and protein levels in these adipocytes in response to glyceollins, therefore revealing an intracellular target of glyceollin stimulation.

Glucose uptake by adipocytes is dependent on the activity of GLUTs. GLUT1 is thought to be responsible for basal glucose uptake by adipocytes and most other cells. GLUT4, expressed by adipose and other insulin target tissues, is responsible for insulin-stimulated glucose uptake. Both GLUTs are expressed by 3T3-L1 differentiated mature adipocytes.[27] In our study, in agreement with the glucose uptake results, the expression of both GLUT1 and GLUT4 was significantly upregulated by glyceollins (1−5 μM) in differentiated 3T3-L1 cells, but only GLUT4 protein levels were significantly increased. These findings offer, for the first time, an insight into the mechanism for glyceollin-mediated glucose uptake by adipocytes. Glyceollins may act in concert with insulin or independently of the hormone by stimulating biosynthesis of the Glut4 transporter protein that promotes transfer of glucose into adipocytes.

Our data suggest that ingestion of soy foods containing glyceollins, in the absence of other soy isoflavones, improves the disposal of circulating glucose via direct stimulation of glucose uptake by glyceollins. Previously, it was proposed that glucose regulation by glyceollins is indirect via stimulation of the insulin signaling pathway.[15] Recently, a downstream event in the insulin signaling pathway—activation of Akt and AMPK—was documented in liver samples isolated from glyceollin-enriched fermented soy-fed diabetic mice.[16] Those signaling events were associated with improvement in the oral glucose tolerance and hepatic glycogen homeostasis.[16] Collectively, glyceollins appear to enhance glucose tolerance directly and in concert with insulin.

Antidiabetic effects of the glyceollins are dependent on many other factors including pharmacokinetics and pharmacodynamics. Little is known about the absorption of the glyceollins into the blood. In a macaque model fed glyceollin-enriched (9 mg/kg) soy protein, glyceollin levels 4 h after oral administration were 134.2 nmol/L (45.4 ng/mL), and <1 nmol/L glyceollin was detected 24 h later.[28] Our results in rats indicated the glyceollins enter the bloodstream by 20 min and achieve a peak concentration (118.4 and 159.0 ng/mL) by 60 min when administered by oral gavage (30 and 90 mg/kg). The difference in exposure levels between the two doses was not linear, suggesting that the 30 mg/kg dose was near maximal. Glyceollin concentrations exhibit the typical absorption phase rise after oral dosing, but the levels do not drop significantly by 240 min. This persistent exposure is unusual for isoflavones; however, it may explain why Park et al. observed similar improvement in an OGTT with a much lower dose (3 mg/kg) of glyceollins.[16] A typical absorption rate with a slow clearance of glyceollin may contribute to an accumulation of glyceollins and produce a consistent exposure to glyceollins in rodents, especially if dosed by dietary admixture. In addition, there could be interactions of the glyceollins with other isoflavones, as well as with the chow, that affect pharmacokinetics of glyceollins when dosing with a fermented soy product rather than with isolated and purified compounds. Results of our glyceollin exposure study—the first to characterize glyceollins bioavailability in a rodent—suggest a prolonged half-life of the compounds.

Prediabetes typically progresses to T2D without intervention. Unfortunately, the first line of treatment is diet and exercise, which usually lacks patient compliance. Thus, safe and effective drugs are being developed for this indication. The possibility of developing unique plant-derived interventions that contain natural compounds and have been consumed safely for thousands of years offers a promising alternative to drugs and lifestyle changes. In the present study, we found that the soy phytoalexin glyceollins were bioavailable in a rat after ingestion and improved the ability to dispose of blood glucose after an oral glucose challenge. We demonstrated that this was due in part to the ability of the glyceollins to stimulate GLUT4 expression in adipocytes and thus increase both basal- and insulin-mediated glucose uptake. Our results provide evidence that the natural plant-derived component glyceollins could be developed into an effective and safe intervention for prediabetes and diabetes.

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Notes

The authors declare no competing financial interest.

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ABBREVIATIONS USED

AUC, area under the blood glucose curve; DMEM, Dulbecco's modified Eagle's medium; DMSO, dimethyl sulfoxide; ESI, electrospray ionization; GLUT, glucose transporter; HEPES, *N*-2-hydroxy ethyl piperazine-*N*′-2-ethane sulfonic acid; HPLC, high-performance liquid chromatography; KRH, Krebs−Ringer−HEPES buffer; OGTT, oral glucose tolerance test; PCR, polymerase chain reaction; PPARγ, peroxisome proliferator activator receptor γ; T2D, type 2 diabetes.

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