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Daidzein and the daidzein metabolite, equol, enhance adipocyte differentiation and PPARγ transcriptional activity

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Abstract

Dietary soy isoflavones have been shown to favorably alter the metabolic phenotypes associated with Type 2 diabetes. However, the identification of direct targets and the underlying molecular mechanisms by which soy isoflaovones exert antidiabetic effects remain elusive. Since the insulin-sensitizing effects of thiazolidinediones, antidiabetic drugs, are mediated through activation of peroxisome proliferators-activated receptor gamma (PPARγ), we examined the effects of daidzein and the daidzein metabolite, equol, on adipocyte differentiation and PPARγ activation. In 3T3-L1 cells, daidzein enhanced adipocyte differentiation and PPARγ expression in a dose-dependent manner. Daidzein also dose-dependently increased insulin-stimulated glucose uptake and the relative abundance of insulin-responsive glucose transporter 4 (GLUT4) and insulin receptor substrate 1 (IRS-1) mRNA. In C3H10T1/2 cells, both daidzein and equol at 1 μmol/L and higher significantly increased adipocyte differentiation and insulin-stimulated glucose uptake. Furthermore, daidzein and equol up-regulated PPARγ-mediated transcriptional activity, and daidzein restored the PPARγ antagonist-induced inhibition of aP2 and GLUT4 mRNA levels. Our results indicate that daidzein enhances insulin-stimulated glucose uptake in adipocytes by increasing the expression of GLUT4 and IRS-1 via the activation of PPARγ. These data further support the recent findings that favorable effects of dietary soy isoflavones may be attributable to daidzein and its metabolite equol.
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Keywords: Daidzein; Equol; PPARγ; Adipocyte differentiation; Insulin sensitivity; GLUT4

1. Introduction

The prevalence of Type 2 diabetes mellitus (T2DM), characterized by hyperglycemia and hyperlipidemia [1–3], has reached epidemic proportions [4]. In the US, the incidence of T2DM has increased by about 33% over the past decade and is expected to increase further [5,6]. Although the pathogenesis of diabetes is complex, insulin resistance, the failure to respond to normal circulating concentrations of insulin, is a major underlying factor contributing to the development of T2DM [7]. Dietary factors that effectively enhance insulin sensitivity represent a novel approach to intervene the development of metabolic disorders such as diabetes and obesity. Therefore, the identification and characterization of natural compounds which could improve insulin sensitivity is important for the treatment and prevention of T2DM.

Soy is a major US agricultural crop that could have important economic and health benefits. Recent clinical and epidemiological studies have shown that soy intake is associated with reduced incidence of diabetes [8] and T2DM-related symptoms [9,10], which has led the Food and Drug Administration to approve cardiovascular health claims for soy protein intake.

Soy isoflavones have been also shown to decrease blood glucose levels and improve glucose tolerance in diabetic animal models [11,12]. Moreover, Jayagopal et al. [13] reported that dietary supplementation with isoflavones reduced insulin resistance and improved glycemic control in T2DM patients and also lowered low-density lipoprotein cholesterol. These studies indicate that soy isoflavones may enhance insulin sensitivity. However, the target(s) of soy isoflavones and the molecular mechanism(s) by which isoflavones sensitize the insulin-responsive tissues remain elusive.

Adipose tissue is an important site of both glucose and lipid metabolism. Insulin resistance is associated with obesity and various syndromes of lipodystrophy [14–16]. Systemic insulin resistance is observed in mice lacking insulin-sensitive glucose transporter 4 (GLUT4) in adipose tissue, indicating that glucose metabolism in adipocytes is critical to whole-body glucose homeostasis [17]. Insulin responsiveness is acquired during the maturation phase of adipocyte differentiation (adipogenesis) and involves the expression of proteins responsible for the phenotypic functions of adipocytes, such as GLUT4 and insulin receptor substrate 1 (IRS-1) [18,19]. Gene expression profiling in adipose tissue demonstrated that several genes related to

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the process of adipocyte differentiation are decreased in Type 2 diabetic humans and animal models of T2DM [20,21], suggesting that altered or impaired adipocyte differentiation may underlie or promote the development of the insulin resistance.

Adipocyte differentiation involves a complex interplay between peroxisome proliferators-activated receptor gamma (PPARγ) and other transcription factors, notably the CCAAT/enhancer binding proteins and adipocyte determination and differentiation factor-1/sterol regulatory element-binding protein-1 [22]. Among them, PPARγ has been considered as an essential regulator of adipocyte differentiation [23–25]. In addition to its regulatory role in adipogenesis, PPARγ activation modulates the expression of several key molecules involved in insulin signaling, lipid metabolism and endocrine function in adipocytes (16). It is well known that PPARγ activation mediates the anti-diabetic activities of thiazolidinediones (TZDs) [26,27]. Although PPARγ is expressed in several tissues, a crucial role of PPARγ in adipose tissue is suggested by the observation that mice lacking PPARγ in adipose tissue are resistant to the antidiabetic effects of TZDs [28].

Previously, we found that dietary isoflavones increase PPARγ and GLUT4 protein in adipose tissue and reduced the severity of diabetes in Zucker diabetic fatty rats [12]. Given that adipocyte differentiation plays a key role in the insulin resistance, and given the potential clinical application of soy isoflavones as insulin sensitizers, we hypothesized that soy isoflavones exert antidiabetic effects through a mechanism that involves PPARγ activation during adipocyte differentiation. Daidzein is one of major soy isoflavones, and equol is a metabolite of daidzein that is proposed to be an important bioactive metabolite of isoflavones [38]. Here, we examined the effects of daidzein and equol on adipocyte differentiation by assessing responsiveness to insulin and regulation of PPARγ, GLUT4 and IRS-1 expression.

2. Materials and methods

2.1. Materials

Daidzein, bisphenol A diglycidyl ether (BADGE) and GW9662 were purchased from Sigma (St. Louis, MO, USA). Equol was a gift from Dr. Connie Weaver (Purdue University, West Lafayette, IN, USA). Anti-PPARγ and anti-actin antibodies were from Santa Cruz Biotechnology (Santa Cruz, CA, USA). [1-^{3}H]2-deoxyglucose (12 μCi/mmol), [α-^{32}P]dCTP (3000 μCi/mmol), enhanced chemiluminescence kit and nylon membranes were from Amersham Pharmacia Biotech (Piscataway, NJ, USA).

2.2. Cell culture of 3T3-L1 and C3H10T1/2 cells

Murine 3T3-L1 preadipocytes were cultured in DMEM/high-glucose medium containing 10% calf serum until confluent (Day −2) and maintained for an additional 2 days (until Day 0). Differentiation was induced on Day 0 by the addition of 0.5 mmol/L methylisobutylxanthine, 1 μmol/L dexamethasone, 1 μg/ml insulin and 10% fetal bovine serum (FBS) in DMEM. After 48 h (Day 2), the medium was replaced with DMEM containing 1 μg/ml insulin and 10% FBS. Medium was changed every 2 days thereafter until the cells were collected for analysis [29]. C3H10T1/2 cells (10T1/2 cells) were maintained in DMEM media and differentiated as described previously [30]. Upon reaching confluence, cells were induced to differentiate in DMEM supplemented with 10% FBS and 0.5 mmol/L methylisobutylxanthine, 1 μmol/L dexamethasone, 10 μg/ml insulin and 1 μmol/L BRL49653 for 2 days. Cells were then incubated in DMEM (Dulbecco's modified eagle medium) containing 10% FBS and 10μg/ml insulin until the cells were collected for analysis. Daidzein and equol were reconstituted as 100 mmol/L stock solutions in DMSO (dimethyl sulfoxide) and added at the indicated concentrations on Day 0. Cells were cultured with isoflavones until cells were collected for analysis.

2.3. Oil Red O staining and intracellular triacylglycerol measurement

Oil Red O staining was performed as previously described [31]. Briefly, cells were washed three times with phosphate-buffered saline (PBS), fixed in 10% formalin for 1 h at room temperature, washed once with PBS and stained with 60% filtered Oil Red O stock solution for 20 min. Cells were washed three times with water and then photographed.

Cytoplasmic lipid was isolated with isopropanol, and the triacylglycerol content measured with a commercial kit (Wako, Richmond, VA, USA). The triacylglycerol was then normalized to protein content, which was determined by BCA protein Assay Reagent (Pierce, Rockford, IL, USA).

2.4. RNA analysis

Total RNA was isolated with Trizol according to the manufacturer's protocol (Invitrogen, Gaithersburg, MD, USA). Northern blot analysis was performed as described previously [29]. In brief, 20 μg of total RNA was separated by agarose-formaldehyde gel electrophoresis and transferred to nylon membranes. The blot was hybridized with ^{32}P-labeled cDNA probes synthesized using the divergent 5′ or 3′ untranslated regions specific for PPARγ. A β-actin cDNA probe was used as an internal loading control. For the quantitative reverse transcriptase-polymerase chain reaction (RT-PCR), 2 μg of RNA was treated with DNase, and cDNA was prepared using the SuperScript First-Strand Synthesis System according to the manufacturer's instructions (Invitrogen). Quantitative RT-PCR was performed using an iCycler (Bio-Rad), and gene expression was measured using SYBR-green and gene-specific primer sets [32]. Target gene expression was normalized to either 36B4 or GAPDH (glyceraldehyde-3-phosphate dehydrogenase) expression. The specificity of the PCR amplification was verified by melt-curve analysis. Reaction products were visualized by agarose gel electrophoresis and ethidium bromide staining.

2.5. Immunoblotting analysis

Cells were lysed in 0.5 ml lysis buffer [10 mmol/L Tris-HCl pH 7.6, 5 mmol/L EDTA, 50 mmol/L NaCl, 30 mmol/L sodium pyrophosphate, 50 mmol/L NaF, 0.1 mmol/L Na_3VO_4, 1% Triton X-100, 1 mmol/L PMSF (phenylmethylsulphonyl fluoride) and protein inhibitor cocktail table (Roche, Indianapolis, IN, USA)] on Day 8 after induction of differentiation. Lysates were clarified by centrifugation at 15,000×g for 10 min. One hundred micrograms of protein was separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and transferred to membrane. Membranes were blocked with 5% nonfat milk in Tris-buffered saline containing tween 20 (TBST) for 1 h and incubated with anti-PPARγ or with anti-actin antibody in 5% nonfat milk in TBST. Blots were washed with TBST, incubated with goat anti-mouse IgG conjugated with horseradish peroxidase, and visualized by enhanced chemiluminescence (Amersham Pharmacia Biotech). PPARγ expression was normalized to β-actin.

2.6. 2-Deoxyglucose uptake measurements

The rate of glucose transport was determined by measuring the uptake of 2-deoxy-[^{3}H]glucose (12 μCi/mmol, Amersham Bioscience) as described [33]. Briefly, cells were grown in six-well plates and differentiated in the presence or absence of isoflavones. At Day 5, the cells were washed once with warm PBS; incubated in serum-free, low-glucose DMEM for 3 h at 37°C and then stimulated with either 100 nmol/L insulin for 10 min at 37°C in KRP (Kreb's ringer phosphate) buffer (128 mmol/L NaCl, 4.7mmol/L KCl, 1.25 mmol/L $CaCl_2$, 1.25 mmol/L $MgSO_4$, 10 mmol/L NaPO4, pH 7.4). After preincubating adipocytes with KRP buffer in the presence of insulin, 2-deoxy-[^{3}H]glucose (5 μCi/well) was added. Uptake was stopped after 5 min by washing the cells three times with cold PBS buffer. A correction for non-carrier-mediated uptake was made by measuring the uptake of [1-^{3}H]-2-deoxyglucose in the presence of 20 μmol/L cytochalasin B, a nonspecific inhibitor of glucose uptake.

2.7. Transfection and luciferase assays

3T3-L1 cells were grown to ≈ 95% confluence in DMEM supplemented with 10% FBS and 1% penicillin-streptomycin. Cells were then transiently cotransfected with a plasmid containing the luciferase gene under control of three tandem PPAR response element (PPRE) (PPRE×3 TK-luciferase) and mouse PPARγ expression plasmids (a gift from Dr. Evan Rosen) using a FuGene 6 transfection reagent according to the manufacturer (Roche). In all cases, a β-galacotsidase expression plasmid was cotransfected to control for transfection efficiency [34]. After the transfection (48 h), cells were cultured for another 24 h in the medium containing DMSO, daidzein or equol. Cells were then washed twice with PBS and lysed in lysis buffer (Promega, Madison, WI, USA). Luciferase activity was measured and expressed as fold induction±S.E.M., which was corrected for transfection efficiency using β-galactosidase activity. Each condition was performed with $n \geq 4$ for each experiment. All experiments were repeated at least three times.

2.8. Statistical analysis

Data are presented as means±S.E.M. Differences between groups were accepted at $P<.05$, which were determined by one-way analysis of variance and post hoc comparisons (LSD; least significant difference) using SAS 6.1.

3. Results

3.1. Effects of daidzein and equol on adipocyte differentiation

To determine whether daidzein affects adipocyte differentiation, 2-day post confluent 3T3-L1 preadipocytes were induced to differentiate in the presence and absence of varying doses of daidzein. As shown in Fig. 1, daidzein dose-dependently increased adipoyte

differentiation at concentrations between 0 and 100 μmol/L. To examine whether physiological concentrations of daidzein and equol, a daidzein metabolite, would affect adipocyte differentiation, multipotent 10T1/2 cells were induced to differentiate in the presence and absence of varying doses of daidzein and equol. Intracellular TAG concentration was dose-dependently increased in cells treated with daidzein between 1 and 20 μmol/L (Fig. 2B). Equol also significantly enhanced adipocyte differentiation of 10T1/2 cells in a range from 0.1 to 20 μmol/L compared to vehicle (Fig. 2).

3.2. Correlation of adipocyte differentiation and PPARγ mRNA expression

Since PPARγ plays a key role in adipocyte differentiation, we determined whether the changes in response to daidzein and equol (Figs. 1 and 2) correlate with changes in the expression of PPARγ expression during the differentiation of 3T3-L1 and 10T1/2 cells. PPARγ mRNA expression was significantly enhanced in 3T3-L1 cells differentiated with MDI plus daidzein in a dose-dependent manner at a concentration between 0 and 20 μmol/L, and it reached a plateau at 20 μmol/L (Fig. 3A). Consistent with the result obtained in 3T3-L1 cells, both daidzein and equol dose-dependently enhanced PPARγ mRNA levels during the adipogenic differentiation of 10T1/2 cells (Fig. 3B). Similarly, daidzein dose-dependently up-regulated PPARγ protein expression up to 20 μmol/L with no significant difference between 1 and 10 μmol/L, while equol significantly increased PPARγ protein level at 1 μmol/L and then reached a plateau as shown in Fig. 3C.

3.3. Comparison of basal and insulin-stimulated 2-deoxyglucose transport by daidzein and its metabolite equol

Because PPARγ is the molecular target for TZD antidiabetic drugs that improve insulin sensitivity and glucose tolerance, we examined whether the increased adipocyte differentiation by daidzein and daidzein metabolite, equol, results in enhanced insulin-stimulated glucose uptake. As shown in Fig. 4A, insulin-stimulated glucose uptake was significantly increased in cells treated with 20 or 100 μmol/L daidzein, whereas only 100 μmol/L of daidzein significantly enhanced basal glucose uptake. In the 10T1/2 cells, daidzein and equol significantly increased insulin-stimulated glucose uptake at 1 μmol/L concentration, and this increase was ~173% and ~132% at 10 μmol/L, respectively.

3.4. Effects of daidzein on the GLUT4 and IRS-1 mRNA

To understand the underlying mechanism for the effects of daidzein on 2-deoxyglucose uptake, we examined the expression of the genes encoding GLUT4 and IRS-1, which are the key downstream signaling molecules involved in insulin-stimulated glucose uptake in adipocytes. The expression of GLUT4 and IRS-1 mRNAs were significantly up-regulated in cells exposed to 5–50- μmol/L daidzein compared to control cells (Fig. 5). GLUT4 and IRS-1 mRNA abundance was increased by ~4.6- and ~2.5-fold, respectively, in cells treated with 20 μmol/L daidzein compared to the control.

3.5. Effects of daidzein on PPARγ transcriptional activity

To investigate whether PPARγ activation would be involved in isoflavone-induced effects on adipocyte differentiation, we first examined the effects of isoflavones on PPARγ transcriptional activity using a reporter gene assay. 3T3-L1 cells were transiently cotransfected with a PPAR-regulated luciferase plasmid containing three PPREs and a PPARγ expression plasmid. PPAR-regulated luciferase expression was measured following treatment with varying concentration of daidzein and equol for 24 h. As shown in Fig. 6, both daidzein and equol at 10 μmol/L significantly increased PPARγ transcriptional activity in 3T3-L1 cells. At 20 μmol/L, both compounds significantly increased PPARγ transcriptional activity by 4- and ~6.5-fold, respectively.

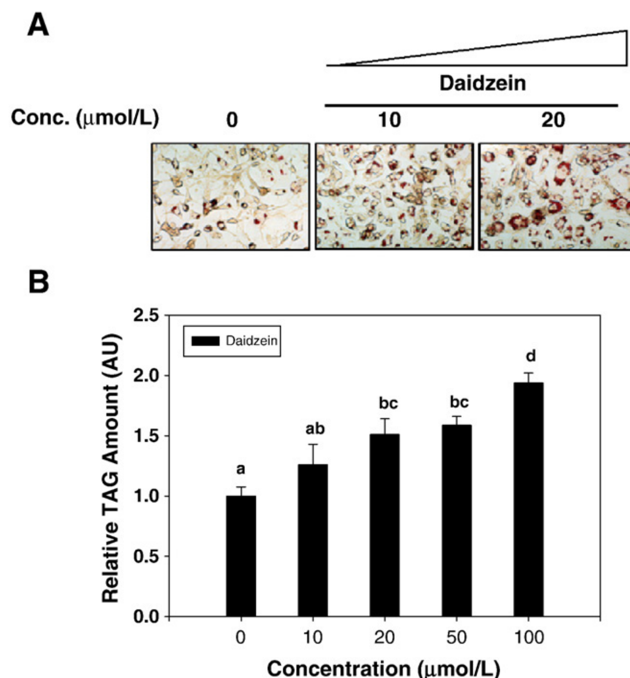

Fig. 1. Effect of daidzein on adipocyte differentiation of 3T3-L1 cells. 3T3-L1 preadipocytes were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations (0, 20, 50, and 100 μmol/L) of daidzein throughout the differentiation for 5 days. (A) The lipid content of cells was evaluated by Oil Red O staining, and (B) measurement of triglyceride. The average TAG amount in vehicle treated cells was 0.12 mg TAG/mg protein. Means±S.E.M. from three different plates is shown and the results is representative of at least five different experiments. Means without common letters are significantly different (P<.05).

Since expression of aP2 and GLUT4 mRNA reflect adipocyte differentiation and glucose uptake in 3T3-L1 cells (22), we determined aP2 and GLUT4 mRNA levels in the presence of daidzein alone, PPARγ antagonist alone or both. As shown in Fig. 7, 20 and 50 μmol/L BADGE, a reversible PPARγ antagonist, effectively inhibited the expression of aP2 and GLUT4, markers for adipocyte differentiation and glucose uptake in 3T3-L1 cells. In the presence of 20 μmol/L BADGE, daidzein restored the BADGE induced-inhibition of aP2 and GLUT4 gene expression, suggesting the requirement of PPARγ activation for the daidzein-mediated glucose uptake. However, there was no difference in aP2 and GLUT4 mRNA levels between BADGE alone and the combination of daidzein and BADGE when 50 μmol/L BADGE was used.

4. Discussion

Epidemiological data and intervention studies in humans have demonstrated that higher intake of dietary soy isoflavones correlate with lower incidence of diabetes and improved insulin sensitivity (9, 13). In vitro cell culture studies also show that soy isoflavones modulate activation of PPARγ, a nuclear transcription factor and receptor for antidiabetic drugs, and expression of its downstream target genes [11,35]. However, the positive effects on PPARγ-directed gene expression are observed when using higher than physiological concentrations in cell types that do not necessarily represent primary expression and activity sites of PPARγ in vivo. It is also uncertain whether potential benefits are mediated by the additive effects of

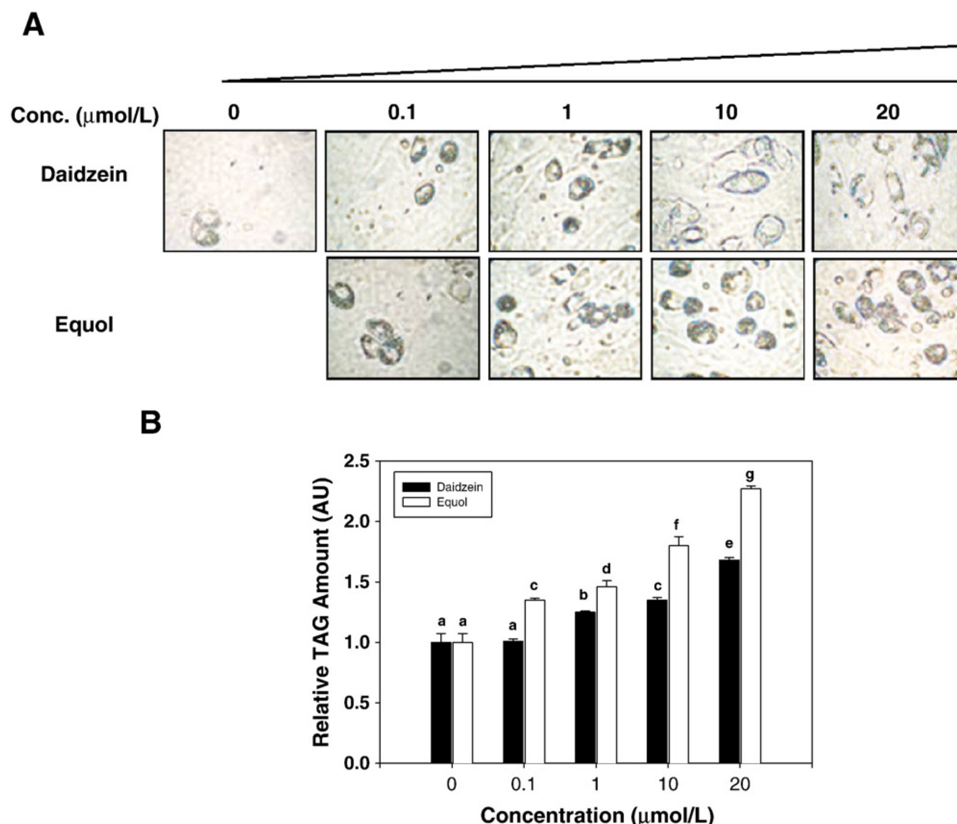

Fig. 2. Effect of daidzein and equol on the adipocyte differentiation of 10T1/2 cells. 10T1/2 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of daidzein and equol (0, 0.1, 1.0, 10 and 20 μmol/L) throughout differentiation for 8 days. (A) Cell morphology was monitored under a phase-contrast microscope and (B) intracellular triglyceride of cells was measured. The values are expressed as fold changes, compared with control (vehicle), which was arbitrarily set to 1. Each value is the Mean±S.E.M. of the results from three different plates and is representative of results from at least five different experiments. Means without a common letters are significantly different (P<.05).

multiple isoflavones components or the concentration of a single isoflavone because most clinical studies have used soy protein containing several different isoflavones.

Genistein and daidzein are the major isoflavones found in soy [36]. Although they have similar structure, they have been shown to have differential effects on tyrosine kinase activity and antioxidant activity [37,38]. After ingestion of soy isoflavones, these compounds could be absorbed in aglycone form or metabolized to biochanin A and equol by intestinal bacteria [39]. Recently, it was suggested that equol, a metabolite of daidzein, may be a more important bioactive metabolite because of its greater binding affinity to estrogen receptors than its parent compound. Thus, an individual's responsiveness to isoflavones may vary according to inherent equol-synthesizing capacity [39–42]. It is unclear whether individual daidzein and/or equol would have same property in adipocyte differentiation. Here we demonstrated that daidzein dose-dependently stimulated adipogenesis using two cell types; a well-established in vitro model of adipocyte differentiation, the 3T3-L1 cell line and pluripotent 10T1/2 cells that are more sensitive and thus more responsive to physiological doses. The effects of daidzein on adipocyte differentiation were comparable with regard to morphological changes, biochemical (triacylglycerol amount) and molecular (gene expression) responses (Figs. 1–3). Equol also enhanced the conversion of 10T1/2 cells to adipocytes and increased PPARγ expression in a dose-dependent manner; this stimulatory effect of equol was greater than that of daidzein (Fig. 2).

Increased insulin-stimulated glucose uptake occurs in differentiating adipocytes, but does not completely correlate with morphological and biochemical changes of adipocyte differentiation [43]. El-Zack et al. [43] showed that PPARγ overexpression in NIH3T3 cells enhanced lipid accumulation but failed to confer insulin sensitivity, due to the lack of GLUT4 expression. Since insulin-stimulated glucose transport is regulated by expression of several key insulin signaling molecules such as insulin receptor substrates (IRS-1 and IRS-2) and GLUT4 [44], we have examined whether daidzein and equol would affect glucose uptake accompanied by alteration of adipocyte differentiation. In parallel to an increment of adipocyte differentiation, daidzein as well as equol significantly increased insulin-stimulated glucose uptake in a dose-dependent manner (Fig. 4). In addition, daidzein increased expression of IRS-1 and GLUT4 mRNAs (Fig. 5), indicting that the enhanced insulin-stimulated glucose uptake by daidzein is mediated in part by up-regulation of IRS-1 and GLUT4 expression.

Consistent with our data, Dang et al [35] also reported that daidzein at concentrations of 30 μmol/L or higher increased adipocyte differentiation using mouse osteoprogenitor KS 483 cell and mouse bone marrow cells. However, Harmon et al. [45] did not observe this stimulatory effect on adipocyte differentiation with 100 μmol/L daidzein, which might be due to the differences of exposure time (3 vs. 5 days), concentrations and/or experimental conditions. In contrast, the anti-adipogenic effects of genistein, a structurally similar to daidzein, have also been reported. Genistein at both 50 and 100 μmol/L has been shown to inhibit adipogenesis of 3T3-L1 cells [45,46], presumably through its inhibitory effects on tyrosine phosphorylation of multiple signaling molecules including p38 and JAK/STAT signaling pathways. Although several reports clearly indicate some effects of soy isoflavones on adipocyte differentiation, there is little information about the effects of

equol, a more biological active metabolite of daidzein, on adipocyte differentiation. To our knowledge, this is the first report to show the dose-dependent effects of daidzein and equol on adipocyte differentiation and functional property of adipocytes linked to insulin sensitivity.

PPARγ is known to be a key mediator of insulin-sensitizing actions of the anti-diabetic drugs. PPARγ is also induced in early stage of adipogenesis and plays a crucial role in adipogenic determination and differentiation [24,25]. In the current study, daidzein and equol dose-dependently enhanced the expression of PPARγ and its target gene aP2 as well as PPARγ transcriptional activity (Figs. 3 and 6). These results indicate that the stimulatory effect of soy isoflavones on adipocyte differentiation may be mediated via up-regulation of PPARγ

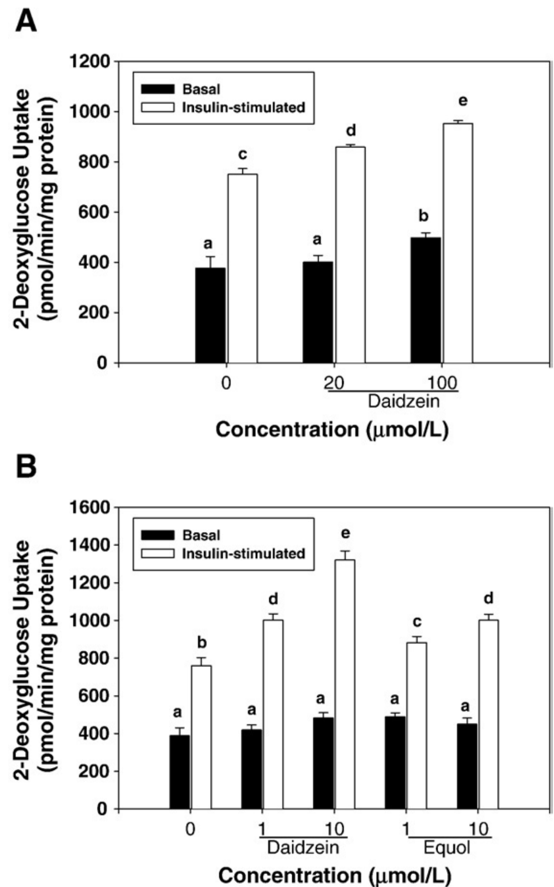

Fig. 4. Effect of daidzein and equol on basal and insulin-stimulated glucose uptake during adipocyte differentiation. (A) Glucose uptake in 3T3-L1 adipocytes treated with daidzein and equol. 3T3-L1 cells were differentiated in the presence of varying concentration of daidzein (0, 20 and 100 μmol/L). At day 5 after the induction of differentiation, basal (black bar) and insulin-stimulated (white bar) glucose uptake was evaluated using 2-deoxy-[^{3}H] glucose as described in Materials and Methods. (B) Glucose uptake in 10T1/2 cells treated by daidzein and equol. 10T1/2 cells were differentiated in the presence of daidzein or equol at indicated concentration for 8 days. After 4 h of serum deprivation, basal and insulin-stimulated (100 nmol/L) glucose uptake was determined using 2-deoxy-[^{3}H] glucose. Non-specific binding activity was corrected using cytochalasin B (20 μmol/L) and specific uptake expressed as picomoles per mg of protein per min. Each value is the mean±S.E.M of the results from three different plates and data are representative of results from at least three different experiments. Different letters indicate statistically significant differences between means at $P<.05$.

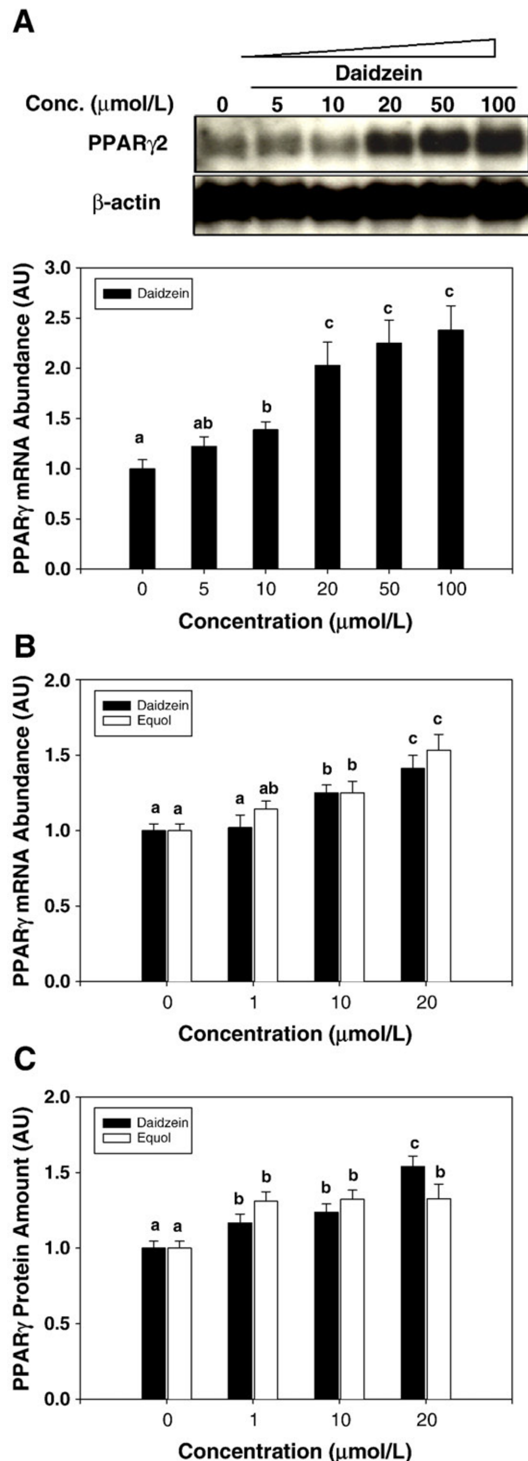

Fig. 3. Effect of daidzein and equol on PPARγ expression during adipocyte differentiation of 3T3-L1 and 10T1/2 cells. (A) PPARγ mRNA in 3T3-L1 cells treated with daidzein and equol. 3T3-L1 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentration of daidzein (0, 5, 10, 20, 50 and 100 μmol/L) throughout differentiation for 5 days. Total RNA was extracted and subjected to Northern blot analysis. Images are representative of three independent experiments. Densitometric data from three separate experiments are plotted as relative abundance of mRNA normalized to β-actin mRNA. (B) PPARγ mRNA expression in 10T1/2 cells treated with daidzein and equol. 10T1/2 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of daidzein and equol throughout differentiation (0, 1, 10, 20 μmol/L) for 8 days. Total RNA was extracted at day 8 and subjected to real-time RT-PCR. PPARγ mRNA levels were quantified and normalized with 36B4 mRNA. (C) PPARγ protein in cells treated with daidzein and equol. 10T1/2 cells were grown and differentiated with the differentiation cocktail in the absence and presence of varying concentrations of equol and daidzein (0, 1, 10 and 20 μmol/L). Cell lysates were immunoblotted with PPARγ antibody. Protein levels of PPARγ were normalized by β-actin protein level. Data are presented as the mean±S.E.M. of 3 experiments. Means without common letters are significantly different ($P<.05$).

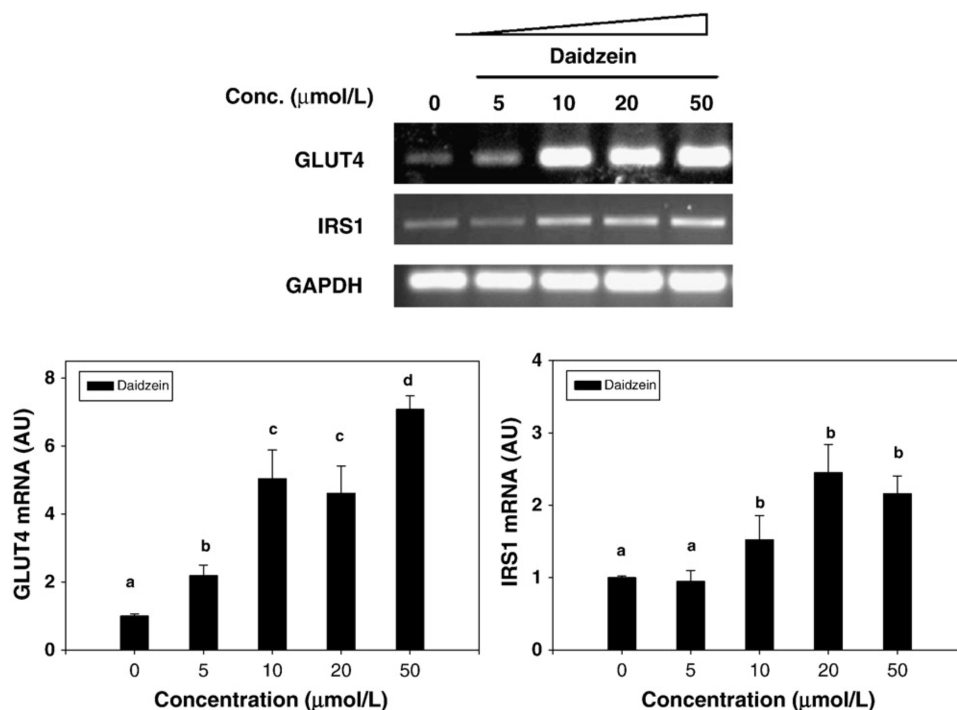

Fig. 5. Effect of daidzein on GLUT4 and IRS-1 mRNA expression during the differentiation of 3T3-L1 cells. 3T3-L1 cells were differentiated in the presence of varying concentration of daidzein (0, 5, 10, 20 and 50 μmol/L) throughout differentiation. At day 5, total cellular RNA was extracted and subjected to RT-PCR. GLUT4 and IRS-1 m RNA levels were quantified and normalized to GAPDH. Images was visualized by Gel-Dot (Bio-Rad, CA) and are representative of three independent experiments (upper panel). Values are expressed as fold change, compared to vehicle, which was arbitrarily set to 1. Data are presented as the mean±S.E.M of 3 experiments (lower panel). Different letters indicate statistically significant differences between means at *P*<.05.

expression as well as its activity. Furthermore, daidzein mimicked the effects of the PPARγ agonist (Fig. 7) by restoring the PPARγ antagonist-induced inhibition of aP2 and GLUT4 expression. Collectively, these results further support the hypothesis that PPARγ is a mediator for the enhanced differentiation by daidzein. However, is remains to be explored how isoflavones would affect PPARγ activity.

The beneficial effects of soy isoflavones on diabetes have been observed when the intake of soy isoflavones was more than 100 mg/day [9,12,13]. Plasma isoflavones concentrations of 1–5 μmol/L have been reported in various population groups consuming foods rich in soy isoflavones [47]. However, a nontoxic high dose of isoflavones was also shown to elevate serum isoflavone concentrations in humans over the 10 μmol/L range [48]. In view of its relevance to humans, the effects of daidzein on adipocyte differentiation and glucose uptake were observed with higher concentrations (≥10 μmol/L) in 3T3-L1 cell line, whereas 1–10 μmol/L daidzein was effective in the mesenchymal stem cell 10T1/2. This suggests that 3T3-L1 cell line requires much higher doses to exert equipotent in vivo effects than the mesenchymal 10T1/2 stem cell line. In the

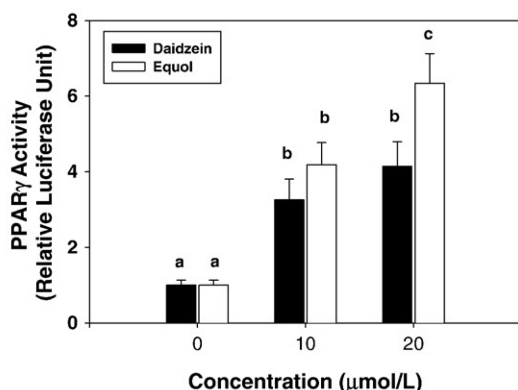

Fig. 6. Effect of daidzein and equol on PPARγ activation in 3T3-L1 cells. 3T3-L1 preadipocytes were transfected with PPRE-containing reporter plasmid, PPARγ expression plasmid and a β-gal expression plasmid. Cells were treated with vehicle or 10 or 20 μmol/L daidzein or equol for 24 h. Cells were harvested and assessed for luciferase and β-galactosidase activities. The data are expressed as induction of luciferase by daidzein and equol relative to control (vehicle). All transfections were performed in triplicates and repeated 3-4 times. Means without common letters are significantly different (*P*<.05).

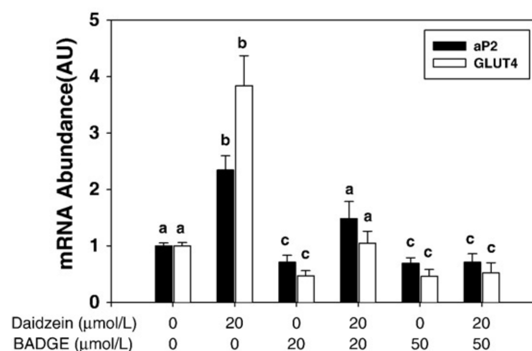

Fig. 7. PPARγ antagonist BADGE inhibits daidzein-induced up-regulation of aP2 and GLUT4 mRNA expression during 3T3-L1 adipocyte differentiation. Confluent 3T3-L1 cells were pretreated with BADGE or vehicle for 24 h and then were differentiated by hormonal cocktail plus vehicle or 20 μM daidzein in the presence or absence of BADGE. On day 5, RNA samples were prepared and real-time quantitative RT-PCR was performed to determine the levels of aP2 and GLUT4 mRNA. The aP2 and GLUT4 mRNA levels were quantified and normalized with the housekeeping gene 36B4, as an internal loading control. The values obtained for the signals are expressed as fold changes compared with control, which was arbitrarily set to 1. Data are representative of results from at least three different experiments. Means without common letters are significantly different (*P*<.05).

previous study, we found that dietary isoflavones increase PPARγ and GLUT4 protein expression in adipose tissue of Zucker Diabetic Fatty rats, thus improving the diabetic symptoms [12]. This in vivo data further support that enhancement of GLUT4 and PPARγ expression by daidzein in adipocytes contribute to the observed anti-diabetic effects of dietary soy isoflavones.

In summary, our results demonstrate that the isoflavone daidzein and its metabolite equol enhance adipocyte differentiation through PPARγ activation. The downstream responses to PPARγ activation include increases in the expression of GLUT4 and IRS-1 mRNA, resulting in enhanced glucose uptake and insulin sensitivity. We also visually observed that daidzein treatment resulted in the generation of small adipocytes, an effect similar to TZD-induced insulin sensitivity. Although the magnitude and alteration of cellularity by daidzein including a redistribution of fat between different adipose depots in vivo remain to be determined, our data provides an additional molecular basis for the mechanism(s) of insulin-sensitizing action by soy isoflavones. These new findings help fill a critical gap between epidemiological observations and clinical studies on the anti-diabetic benefits of dietary soy.

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