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6-Hydroxydaidzein Enhances Adipocyte Differentiation and Glucose Uptake in 3T3-L1 Cells

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ABSTRACT: Fermented soybean foods have been shown to reduce incidence of diabetes and improve insulin sensitivity. 6-Hydroxydaidzein (6-HD) is a bioactive ingredient isolated from fermented soybean. In this study, we examined the effects of 6-HD on adipocyte differentiation and insulin-stimulated glucose uptake, as well as the mechanisms involved. In our experiments, 6-HD enhanced 3T3-L1 adipocyte differentiation and insulin-stimulated glucose uptake in a dosage-dependent manner. In addition, 6-HD increased peroxisome proliferator-activated receptor gamma (PPARγ) gene expression and PPARγ transcriptional activity. 6-HD increased CCAAT/enhanced binding protein alpha (C/EBPα) expression as well. Although having no effects on glucose transporter type 4 (GLUT4) gene expression, 6-HD facilitated GLUT4 protein translocation to the cell membranes. Our results indicate that 6-HD exhibited the actions of promoting adipocyte differentiation and improving insulin sensitivity by increasing the expression of C/EBPα and facilitating the translocation of GLUT4 via the activation of PPARγ, suggesting that 6-HD can be promising in diabetes management.

KEYWORDS: *6-hydroxydaidzein, PPARγ, adipocyte, insulin sensitivity, GLUT4, glucose uptake*

INTRODUCTION

Type 2 diabetes is a metabolic disorder characterized by hyperglycemia and insulin resistance. It is caused by multifactorial etiology, including environmental factors, particularly dietary and genetic components. 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 type 2 diabetes.[1] Adipose tissue is an important site of both glucose and lipid metabolism. Altered or impaired adipocyte differentiation may promote the development of insulin resistance, ultimately resulting in type 2 diabetes.[2,3]

Peroxisome proliferator-activated receptors (PPARs), a subfamily of nuclear hormone receptors, regulate transcription by interaction with the corresponding ligand. Furthermore, PPARγ has the highest expression levels in adipose tissue compared with that of other metabolic organs, such as the skeletal muscle, liver, and pancreas,[4] and it plays a key role in regulating insulin sensitivity and glucose homestasis.[5] Cross-regulation of PPARγ and CCAAT/enhancer binding protein alpha (C/EBPα) controls the transcriptional pathway of adipogenesis and insulin sensitivity.[6] 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.

Thiazolidinediones (TZDs), also known as glitazones, are a class of medications used in the treatment of type 2 diabetes. TZDs are synthetic PPARγ ligands, which stimulate adipocyte differentiation and enhance insulin sensitivity by stimulating the transcriptional activity of PPARγ.[7] However, edema is a main side effect of these drugs, particularly when combined with insulin.[7] Recently, dietary factors that effectively enhance insulin sensitivity without any side effects represent a novel approach to intervene in the development of metabolic disorder such as diabetes and obesity. Therefore, the identification and characterization of bioactive compounds in foods which could improve insulin sensitivity is important for the treatment and prevention of type 2 diabetes.

Soy foods are beneficial for improving insulin sensitivity and reducing the incidence of type 2 diabetes. The evidence does suggest that, compared with nonfermented soybeans, fermented soy products may be better for preventing or delaying the progression of type 2 diabetes, despite the lack of clinical evidence.[8] Furthermore, recent studies show that isoflavonoids and peptides from fermented soybeans had better insulin-sensitizing and antidiabetic actions than did unfermented soybeans.[9,10] However, the exact active ingredients, produced during fermentation, responsible for the beneficial effects on glucose metabolism, and the molecular mechanisms involved remain to be determined. 6-Hydroxydaidzein (6-HD, Figure 1), also named 6,7,4′-trihydroxyisoflavone, is an isoflavone

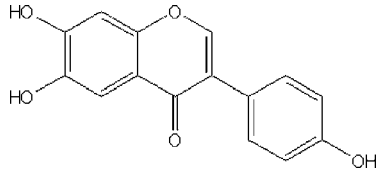

Figure 1. Chemical structure of 6-hydroxydaidzein.

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derivative. It has been isolated from tempeh, a kind of fermented soybean food, but not detected in soybean and seemed to be produced during the fermentation of soybean.[11] 6-HD is also an *in vivo* hydroxylated metabolite of daidzein by the action of the phase II enzyme cytochrome P450 (CYP1A2) in the human liver.[12] It has been reported that 6-HD processes antioxidative[13] and antimutagenic[14] as well as antityrosinase activities.[15] Recently, it was suggested that 6-HD, a hydroxylated metabolite of daidzein, may be a more important bioactive ingredient because of its greater PPARγ binding and transcriptional activities than its precursor.[16] As PPARγ plays a very important role in glucose and lipid metabolism, it would be of interest to determine whether 6-HD is involved in the insulin-sensitizing and the antidiabetic actions of fermented soybeans. Accordingly, we investigated the regulatory effects of 6-HD on adipocyte differentiation and insulin-induced glucose uptake in 3T3-L1 cells along with the mechanisms involved.

MATERIALS AND METHODS

Materials and Reagents. 6-Hydroxydaidzein (6-HD) was isolated from fermented soybeans according to the method described previously.[11] Anti-PPARγ, anticadherin, anti-GLUT4, and anti-β-actin antibodies were purchased from Santa Cruz Biotechnology (Santa Cruz, CA, USA). 3T3-L1 cell lines were obtained from the Cell Bank of China Science Academy (Shanghai, China). Dulbecco's modified Eagle's medium (DMEM) was purchased from Gibco (Gaithersburg, MD, USA). Rosiglitazone (ROZ), dexamethasone (DEX), 3-isobutyl-1-methylxanthine(IBMX), insulin, and Oil Red O were purchased from Sigma-Aldrich (St. Louis, MO, USA). 2-[*N*-(7-Nitrobenz-2-oxa-1,3-diazol-4-yl) amino]-2-deoxy-D-glucose (2-NBDG) was purchased from Invitrogen (Carlsbad, CA, USA). FuGENE 6 transfection reagent was the product of Roche (Indianapolis, IN, USA) and Steady-Glo Luciferase Assay System of Promega Corporation (Madison, WI, USA).

Cell Culture and 3T3-L1 Preadipocyte Differentiation. Mouse 3T3-L1 preadipocytes were cultured in DMEM medium supplemented with 10% fetal bovine serum (FBS) at 37 °C in a 5% CO_2 humidified atmosphere. 3T3-L1 preadipocyte differentiation was induced according to the protocol described previously.[17] In brief, 3T3-L1 cells at 2 days post confluence (day 0) were stimulated to differentiate in a standard adipogenic differentiation medium (DMEM containing 10% FBS, 0.5 mM isobutylmethylxanthine, 0.25 μM dexamethasone, and 10 μg/mL insulin) for 2 days. The differentiation medium was then replaced with DMEM containing 10% FBS and 10 μg/mL insulin. After 2 days, the medium was replaced again with fresh DMEM containing 10% FBS. During preadipocyte differentiation, 3T3-L1 cells were treated with 6-HD at a concentration of 0, 5, 10, or 20 μM from day 0 to 8, and treated with 2 μM ROZ as a positive control. All assays were conducted in triplicate, and at least three separate assays were performed.

Oil Red O Staining and Quantification of Lipid Accumulation. After differentiation, the 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 then stained with Oil Red O solution for 1 h. After the cells were washed three times with distilled water, the cells were photographed using a microscope. Lipid and Oil Red O were extracted using isopropanol, and absorbance was measured using a spectrophotometer at a wavelength of 520 nm. The absorbance was used to evaluate the content of triglyceride (TG) in 3T3-L1 cells. Relative TG contents (%) was displayed using differentiation medium (DM) treated cells as a standard.

Reverse Transcription–Polymerase Chain Reaction (RT-PCR). Total RNA was isolated using Trizol reagent according to the manufacturer's instructions and quantified by UV absorption at 260 nm using a UV spectrophotometer. Total RNA (4 μg) of each sample was reverse-transcribed into cDNA using the reverse transcription system. The cDNA was amplified with the following specific primers: PPARγ, 5′-CCA GAG TCT GCT GAT CTG CG-3′ (forward); 5′-GCC ACC TCT TTG CTC TGA TC-3′ (reverse); GLUT4, 5′-TAC TCA TTC TTG GAC GGT TC-3′ (forward); 5′-TGA TGT AGA GGT ATC TGG GG-3′ (reverse); C/EBPα, 5′-GGT GCT GGA GTT GAC CAG TG-3′ (forward); 5′-CGG AAT CTC CTA GTC CTG GC-3′ (reverse); and β-actin, 5′-AGG CTG TGC TGT CCC TGT ATG C-3′ (forward); 5′-ACC CAA GAA GGA AGG CTG GAA A-3′ (reverse). The PCR products were separated by electrophoresis in a 1% agarose gel and stained with ethidium bromide.

Preparation of Cell Extracts and Western Blot Analysis. 3T3-L1 cells were differentiated in the presence of varying concentrations of 6-HD for 8 days. For total protein extraction, fully differentiated 3T3-L1 cells were lysed in ice-cold buffer A (1% Triton X-100, 100 mM sodium pyrophosphate, 2 mM phenylmethylsulfonyl fluoride (PMSF), and 50 mM HEPES, pH7.4) and centrifuged at 15,000*g* for 20 min at 4 °C. Supernatants were collected and stored at −80 °C. For subcellular fractionation, cells were lysed in buffer B (0.25 M sucrose, 2 mM EDTA, 1 mM phenylmethylsulfony fluoride (PMSF), and 10 mM Tris-HCl, pH 7.4). The lysates were centrifuged at 750*g* for 15 min, and the supernatants were centrifuged at 12,000*g* for 20 min to isolate the crude plasma membrane fraction as the pellets. Equal amounts of protein from each sample were denatured and subjected to SDS–PAGE and blotted on a PVDF membrane, which was incubated for 2 h at room temperature with blocking buffer (5% nonfat milk and 0.1% Tween 20, in TBS, pH 7.6) and then probed with primary antibodies overnight at 4 °C. After incubation with the appropriate secondary antibodies, the immunoreactive band was detected by an ECL Western blotting detection system (GE Healthcare).

Glucose Uptake Assay. 2-NBDG is a fluorescent glucose analogue. Glucose uptake into 3T3-L1 adipocytes was measured using 2-NBDG as previously described with minor modifications.[18,19] Briefly, 3T3-L1 cells were grown in 96-well fluorescent plates and differentiated as described above. At day 8, the cells were washed once with warm PBS, incubated in serum-free DMEM for 3 h at 37 °C, and then stimulated with either 100 nM insulin for 10 min at 37 °C in Kreb's ringer phosphate buffer (128 mM NaCl, 4.7 mM KCl, 1.25 mM $CaCl_2$, 1.25 mM $MgSO_4$, and 10 mM NaPO4, pH7.4). Glucose uptake was initiated by the addition of 80 μM 2-NBDG to each well. After 1 h, the supernatant was removed. Plates were then rinsed with PBS, and the fluorescence of the samples was monitored at an excitation wavelength of 485 nm and an emission wavelength of 535 nm. To exclude false-positive, cells treated with drugs in the absence of 2-NBDG were measured and taken as the background. The relative fluorescence intensities minus the background were used for subsequent data analysis.

Transfection and Luciferase Assays. For transient transfection, 3T3-L1 cells were seeded in a six-well plate with 10% FBS/DMEM. Mouse PPARγ vector (pCMX-mPPARγ), together with the PPRE-Luc reporter and β-galactosidase plasmids, were cotransfected into 3T3-L1 cells using FuGENE 6 (Roche). After 24 h of transfection, the medium was changed to DMEM containing 10% FBS, and the cells were cultured for a further 24 h. Luciferase activity was determined by using the luciferase assay system (Promega) according to the manufacturer's instructions and then normalized to β-galactosidase activity.

Statistical Analysis. Results are expressed as the mean ± SEM. Statistical analysis was performed using one-way analysis of variance (ANOVA). The intergroup comparisons (post hoc analysis) among the data with equal variances were made by the LSD method, whereas Tamhane's T2 method was used for the data with unequal variances. A P value of <0.05 was considered to be significant.

RESULTS

6-HD Enhanced 3T3-L1 Preadipocyte Differentiation. To determine whether 6-HD affects adipogenesis, 2-day postconfluent 3T3-L1 preadipocytes were induced to differentiate in the presence or absence of varying doses of 6-HD. In addition, ROZ, a PPARγ agonist that has been shown to promote adipocyte differentiation,[7] was employed as a positive control in the study. At 8 days after the initiation of differentiation, lipid accumulation was detected by staining

with Oil Red O. As shown in Figure 2, treatment of 3T3-L1 preadipocytes with 10 or 20 μM 6-HD significantly enhanced

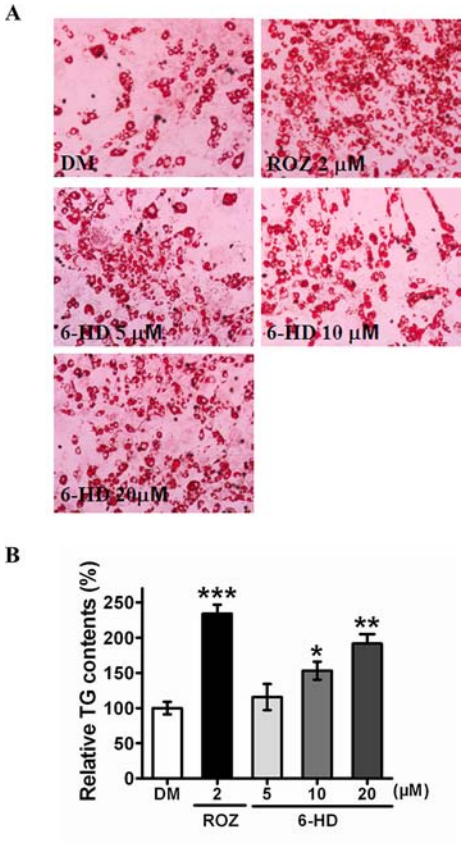

Figure 2. Effects of 6-HD on adipocyte differentiation in 3T3-L1 cells. 3T3-L1 cells were treated with 6-HD (0, 5, 10, or 20 μM) during adipocyte differentiation for 8 days. The control group was induced by standard adipogenic differentiation media (DM) without other treatment. (A) 3T3-L1 adipogenesis was visualized by Oil Red O staining. Representative microscopic pictures were taken at day 8. (B) The stained Oil Red O was extracted with isopropanol. The absorbance of the extracted Oil Red O was measured at a wavelength of 520 nm. TG, triglyceride. Results are presented as the mean ± SEM (n = 3). * P < 0.05, ** P < 0.01, and *** P < 0.001 vs control.

adipocyte differentiation (P < 0.05 and P < 0.01, respectively). The OD value of the Oil Red O eluted solution increased by 1.53- and 1.92-fold in 3T3-L1 cells treated with 10 and 20 μM 6-HD, respectively. Furthermore, ROZ also significantly enhanced adipocyte differentiation at a concentration of 2 μM (P < 0.001).

6-HD Upregulated mRNA and Protein Expression of PPARγ. PPARγ is the master regulator of adipogenesis, thereby stimulating the production of small insulin-sensitive adipocytes.[4] Therefore, we examined whether the changes in response to 6-HD correlate with changes of the expression of PPARγ during the differentiation of 3T3-L1 cells. As shown in Figure 3A, PPARγ mRNA expression was significantly enhanced in 3T3-L1 cells differentiated with standard adipogenic medium plus 6-HD in a dose dependent manner at a concentration between 0 and 20 μM. Similarly, 6-HD dose dependently upregulated PPARγ protein expression (Figure 3B).

6-HD Increased PPARγ Transcriptional Activity in 3T3-L1 Cells. To investigate whether PPARγ activation would be

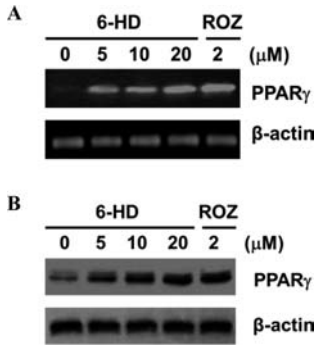

Figure 3. Effects of 6-HD on PPARγ expression in differentiated 3T3-L1 adipocytes. 3T3-L1 cells were grown and differentiated with standard adipogenic medium in the absence or presence of varying concentrations of 6-HD (0, 5, 10, 20 μM) throughout differentiation for 8 days. (A) Total RNA was extracted, and the PPARγ mRNA levels were analyzed by RT-PCR. β-Actin was used as an internal control; n = 3. (B) Protein samples from differentiated adipocytes were prepared, and the PPARγ protein levels were examined by Western blotting. β-Actin was used as an internal control. The results shown are representative of at least three independent experiments.

involved in the effects of 6-HD-induced upregulation of adipocyte differentiation, the effects of 6-HD on the transcriptional activity of PPARγ were evaluated using a reporter gene assay. 3T3-L1 cells were transiently cotransfected with a PPARγ-regulated luciferase plasmid containing three PPREs and a PPARγ expression plasmid. After treatment with 6-HD for 24 h, PPARγ regulated luciferase expression was measured. As shown in Figure 4, 10 μM 6-HD significantly increased

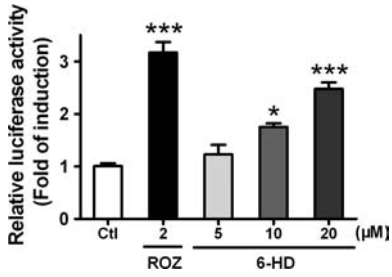

Figure 4. Effects of 6-HD on PPARγ activation in 3T3-L1 cells. 3T3-L1 preadipocytes were cotransfected with PPRE-containing reporter plasmid, PPARγ expression plasmid, and a β-gal expression plasmid. Cells were treated with varying concentrations of 6-HD (0, 5, 10, and 20 μM) for 24 h. Cells were harvested and assessed for luciferase and β-galactosidase activities. The data are expressed as the induction of luciferase by 6-HD relative to the control (vehicle). All transfections were performed in triplicate and repeated 3 times. * P < 0.05 and *** P < 0.001 vs the control.

PPARγ transcriptional activity in 3T3-L1 cells (P < 0.05). Furthermore, at 20 μM, it significantly increased PPARγ transcriptional activity by 2.48-fold.

6-HD Increased Basal and Insulin-Stimulated Glucose Uptake in Differentiated 3T3-L1 Adipocytes. Because PPARγ activation through binding of the synthetic TZDs results in a marked improvement in insulin sensitivity and glucose tolerance,[20] we examined whether the PPARγ activation induced by 6-HD also results in enhanced insulin-stimulated glucose uptake. As shown in Figure 5, insulin-stimulated glucose uptake was significantly increased in cells treated with 6-HD, and this increase was 38%, 57%, and 79% at

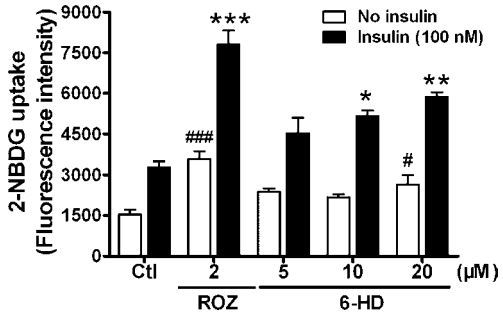

Figure 5. Effects of 6-HD on basal and insulin-stimulated glucose uptake during adipocyte differentiation. 3T3-L1 cells were differentiated in the presence of varying concentrations of 6-HD (0, 5, 10, and 20 μM). At day 8 after the induction of differentiation, basal and insulin (100 nM)-stimulated glucose uptake was evaluated using 2-NBDG as described in Materials and Methods. Values are presented as the mean ± SEM; $n = 3$. $^{\#}$ $P < 0.05$ and $^{\#\#\#}$ $P < 0.001$ vs the basal group; * $P < 0.05$, ** $P < 0.01$ and *** $P < 0.001$ vs the insulin-treated group.

5, 10, and 20 μM, respectively, whereas only at 20 μM concentration, 6-HD significantly enhanced basal glucose uptake ($P < 0.05$).

6-HD Upregulated C/EBPα mRNA Expression but Had No Effects on GLUT4 Gene Expression. As we all know, cross-regulation of C/EBPα and PPARγ controls the transcriptional pathway of adipogenesis and insulin sensitivity.[6] Furthermore, both basal and insulin-stimulated glucose transport in insulin sensitive tissues are closely related to the expression of GLUT4 and its translocation to the plasma membrane induced by PPARγ activation.[21,22] Then, we examined the effects of 6-HD on the expression of GLUT4 and C/EBPα to understand the underlying mechanism of 6-HD for enhancing glucose uptake and adipocyte differentiation. As shown in Figure 6A, the mRNA expression of C/EBPα were

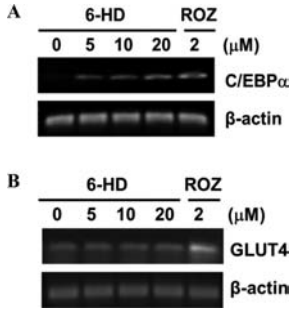

Figure 6. Effects of 6-HD on C/EBPα and GLUT4 mRNA expression during the differentiation of 3T3-L1 cells. 3T3-L1 cells were differentiated in the presence of varying concentrations of 6-HD (0, 5, 10, and 20 μM) throughout differentiation. At day 8, total cellular RNA was extracted. The mRNA levels of (A) C/EBPα and (B) GLUT4 were assessed by RT-PCR. β-Actin was used as an internal control. The results shown are representative of at least three independent experiments.

significantly upregulated in cells treated with 5−20 μM 6-HD compared to that in control cells. However, 6-HD did not significantly increase the GLUT4 mRNA expression, which was apparently different from that of ROZ (Figure 6B).

6-HD Increased the Translocation of GLUT4 to the Membranes. 6-HD did not increase the expression of GLUT4 mRNA in 3T3-L1 adipocytes (Figure 6B). However, it could increase insulin-stimulated glucose uptake in a dose dependent manner (Figure 5). Then, we speculated that 6-HD probably facilitated the translocation of GLUT4 to the membranes or directly increased the synthesis of GLUT4 protein but did not increase mRNA expression. Therefore, the total cellular proteins and the plasma membrane proteins were isolated from 3T3-L1 adipocytes, and Western blot analysis was performed. As shown in Figure 7A, the GLUT4 protein in

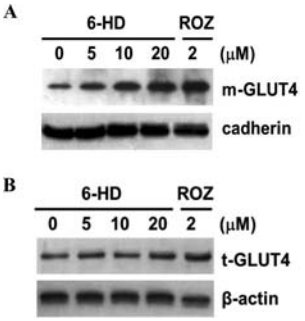

Figure 7. Effects of 6-HD on GLUT4 protein in 3T3-L1 cells. 3T3-L1 preadipocytes were differentiated in the presence of varying concentrations of 6-HD as described above. (A) The plasma membrane GLUT4 protein (m-GLUT4) levels and (B) the total GLUT4 protein (t-GLUT4) levels in 3T3-L1 adipocytes were examined by Western blot. Cadherin and β-actin were used as loading controls for membrane protein or total protein, respectively. The results shown are representative of at least three independent experiments.

the plasma membrane of 3T3-L1 cells treated with 5, 10, and 20 μM 6-HD increased obviously compared to that of the control group, but 6-HD had no effect on the total GLUT4 proteins in adipocytes (Figure 7B). Our results indicated that 6-HD facilitated the translocation of GLUT4 to the membranes, although it did not increase gene expression.

DISCUSSION

Thiazolidinediones (TZDs) are insulin sensitizing drugs used to treat type 2 diabetes. The primary target of the TZDs is the peroxisome proliferator activated receptor gamma (PPARγ), a key regulator of adipogenesis. However, oral antidiabetic drugs, such as TZDs, display undesirable side effects and fail to control the glycemic level effectively.[7] Accordingly, it is highly desirable to find natural antidiabetic alternatives that stimulate adipogenesis and glucose uptake in adipocytes but do not induce obesity or other side effects.

Soy, when absorbed into the bloodstream, exhibits hypoglycemic properties and also provides nourishment to the body. Isoflavones are biologically active compounds that are present in relatively large amounts in soybean. Soy isoflavones have been shown to improve insulin sensitivity and decrease blood glucose in diabetic animal models[23] and type 2 diabetes patients.[24] However, recently, it was reported that fermented soy products may be better for preventing or delaying the progression of type 2 diabetes compared with nonfermented soybeans.[8] Furthermore, isoflavonoids and peptides from long-term fermented soybeans had better insulin-sensitizing actions by activating PPARγ in 3T3-L1 adipocytes than did unfermented soybeans.[9] That is to say, the compositional changes in isoflavonoids and peptides that occurred during a longer fermentation period enhanced the antidiabetic effect of soybeans. However, the exact active ingredients produced

during fermentation, which are responsible for the better antidiabetic action of fermented soybeans than unfermented soybeans, are still unknown, and the molecular mechanisms involved remain to be determined.

6-HD, an isoflavone derivative, was isolated from tempeh, a kind of fermented soybean food.[25] It was reported that bacteria isolated from tempeh biotransformed daidzein to 6-hydroxydaidzein (6-HD).[26] In addition, 6-HD has also been isolated from fermented soybean products.[11,27] However, 6-HD was not detected in soy and seemed to be produced during fermentation by A. *oryzae*.[11] That is to say, 6-HD exists only in fermented soybean but not in soy. Moreover, it was reported recently that 6-HD exerted high PPARγ binding affinity and transactivation potential.[16] The PPARγ binding affinity of 6-HD was more than 100-fold higher than its precursor daidzein, and the maximal induction level of 6-HD on PPARγ activity exceeded even that of rosiglitazone, a known PPARγ agonist in the thiazolidinedione class.[16] Accordingly, we speculated that 6-HD, a hydroxylated metabolite of daidzein, may play a very important role in improving glucose and lipid metabolic homeostasis.

In this study, we used 3T3-L1 preadipocyte cell line, a well-established model of adipocyte differentiation to determine the effects of 6-HD on adipocyte differentiation and insulin-induced glucose uptake. Our results demonstrated that 6-HD promoted adipocyte differentiation significantly after treatment for 8 days at concentrations from 10 to 20 μM (Figure 2). However, Seo et al. have recently reported that 6-HD (6,7,4′-THIF) repressed 3T3-L1 adipogenesis at 40 and 80 μM.[28] As a partial PPARγ agonist exemplified by Mueller et al.[16] or Figure 3, it is not unexpected that 6-HD antagonizes PPARγ transactivation and thus inhibits adipocyte differentiation at high concentrations. Our data clearly showed that 6-HD could antagonize ROZ-induced PPARγ transactivation in a reporter gene assay at high concentrations (data not shown).

We also examined whether these effects are regulated by PPARγ and its downstream target genes, C/EBPα and GLUT4. Moreover, 6-HD has the ability to stimulate glucose uptake far better in the presence of insulin than in the absence of insulin (Figure 5). As we all know, PPARγ is expressed at high levels in adipose tissue, and it plays an important role in the regulation of genes expression involved in adipocyte differentiation, lipid storage, glucose metabolism, and insulin sensitivity. In the current study, 6-HD enhanced PPARγ mRNA and protein expression as well as PPARγ transcriptional activity (Figures 3 and 4). Our results indicate that the stimulatory effect of 6-HD on adipocyte differentiation and insulin sensitivity may be mediated via the upregulation of PPARγ expression as well as its activity. However, how 6-HD would affect PPARγ activity and upregulate PPARγ expression remains to be explored.

It has been suggested that 2.2 g of isoflavone consumption can cause an approximate 80 μM plasma concentration.[28] Although there is a lack of studies into the quantity of 6-HD derived from daidzein intake or the exact metabolic mechanisms of 6-HD *in vivo*, we believe that long-term consumption of isoflavones could lead to a sustained 6-HD blood concentration that exerts antidiabetic effects.

The adipocyte differentiation program is regulated by a coordinated interplay of key transcription factors such as PPARγ and C/EBPα that ultimately regulate the late marker genes and phenotypic functions of an adipocyte.[29] Further study showed that 6-HD significantly increased C/EBPα mRNA expression (Figure 6A).

In mammalian cells, facilitative diffusion of glucose across the plasma membrane is mediated by a family of glucose transporters. GLUT4, one of the glucose transporter isoforms, is only expressed in peripheral tissues that are targets for insulin action, namely, adipose tissue, cardiac muscle, and skeletal muscle. Upon acute stimulation with insulin, GLUT4 translocates from its intracellular compartment to the plasma membrane and therefore is responsible for insulin-stimulated glucose uptake.[21] In the present study, no significant increase was observed for the GLUT4 gene expression in 6-HD treated 3T3-L1 cells, compared with a significant increase by ROZ at a concentration of 2 μM (Figure 6B). However, 6-HD significantly promoted 2-NBDG uptake in 3T3-L1 adipocytes (20 μM 6-HD increased 79% glucose uptake, Figure 5). We then speculated that 6-HD possibly only induced the expression of GLUT4 protein or facilitated the translocation of GLUT4 to the plasma membrane. To verify the presumptions, we used Western blot analysis to examine the effect of 6-HD on the plasma membrane GLUT4 protein or total GLUT4 protein levels. Interestingly, the GLUT4 protein levels in the plasma membrane increased significantly when treated with 6-HD (Figure 7A), but the total GLUT4 protein levels did not (Figure 7B). In view of these findings, we can conclude that 6-HD increased the translocation of GLUT4 to the plasma membrane but did not increase the total protein expression. Furthermore, the mechanisms of insulin sensitization between 6-HD and ROZ were not all the same.

In conclusion, 6-HD enhanced adipocyte differentiation and improved insulin sensitivity through PPARγ activation. The downstream responses to PPARγ activation include increases in the C/EBPα mRNA expression and GLUT4 translocation, resulting in enhanced adipocyte differentiation, glucose uptake, and insulin sensitivity. It is not unexpected that 6-HD may exert these effects on other cell types because of its interaction with PPARγ signaling. To verify the hypothesis, further investigations should be conducted. Taken together, our results provide insights that 6-HD, an isoflavone derivative, isolated from fermented soybean products, has a great potential for regulating adipocyte differentiation and improving insulin sensitivity and that it may have therapeutic potential against diabetes.

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Notes

The authors declare no competing financial interest.

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REFERENCES

(1) Yang, J.; Eliasson, B.; Smith, U.; Cushman, S. W.; Sherman, A. S. The size of large adipose cells is a predictor of insulin resistance in first-degree relatives of type 2 diabetic patients. *Obesity* **2012**, *20*, 932–938.

(2) Guilherme, A.; Virbasius, J. V.; Puri, V.; Czech, M. P. Adipocyte dysfunctions linking obesity to insulin resistance and type 2 diabetes. *Nat. Rev. Mol. Cell Biol.* **2008**, *9*, 367−377.
(3) Danforth, E. J. Failure of adipocyte differentiation causes type II diabetes mellitus? *Nat. Genet.* **2000**, *26*, 13.
(4) Kintscher, U.; Law, R. E. PPARgamma-mediated insulin sensitization: the importance of fat versus muscle. *Am. J. Physiol. Endocrinol. Metab.* **2005**, *288*, E287−291.
(5) Liao, W.; Nguyen, M. T.; Yoshizaki, T.; Favelyukis, S.; Patsouris, D.; Imamura, T.; Verma, I. M.; Olefsky, J. M. Suppression of PPAR-gamma attenuates insulin-stimulated glucose uptake by affecting both GLUT1 and GLUT4 in 3T3-L1 adipocytes. *Am. J. Physiol. Endocrinol. Metab.* **2007**, *293*, E219−227.
(6) Rosen, E. D.; Walkey, C. J.; Puigserver, P.; Spiegelman, B. M. Transcriptional regulation of adipogenesis. *Genes Dev.* **2000**, *14*, 1293−1307.
(7) Ahmadian, M.; Suh, J. M.; Hah, N.; Liddle, C.; Atkins, A. R.; Downes, M.; Evans, R. M. PPARgamma signaling and metabolism: the good, the bad and the future. *Nat. Med.* **2013**, *19*, 557−566.
(8) Kwon, D. Y.; Daily, J. W.; Kim, H. J.; Park, S. Antidiabetic effects of fermented soybean products on type 2 diabetes. *Nutr. Res. (N. Y., NY, U. S.)* **2010**, *30*, 1−13.
(9) Kwon, D. Y.; Hong, S. M.; Ahn, I. S.; Kim, M. J.; Yang, H. J.; Park, S. Isoflavonoids and peptides from meju, long-term fermented soybeans, increase insulin sensitivity and exert insulinotropic effects in vitro. *Nutrition* **2011**, *27*, 244−252.
(10) Kwon, D. Y.; Jang, J. S.; Hong, S. M.; Lee, J. E.; Sung, S. R.; Park, H. R.; Park, S. Long-termconsumption of fermented soybean-derived Chungkookjang enhances insulinotropic action unlike soybeans in 90% pancreatectomized diabetic rats. *Eur. J. Nutr.* **2007**, *46*, 44−52.
(11) Hirota, A.; Inaba, M.; Chen, Y. C.; Abe, N.; Taki, S.; Yano, M.; Kawaii, S. Isolation of 8-hydroxyglycitein and 6-hydroxydaidzein from soybean miso. *Biosci. Biotechnol. Biochem.* **2004**, *68*, 1372−1374.
(12) Atherton, K. M.; Mutch, E.; Ford, D. Metabolism of the soyabean isoflavone daidzein by CYP1A2 and the extra-hepatic CYPs 1A1 and 1B1 affects biological activity. *Biochem. Pharmacol.* **2006**, *72*, 624−631.
(13) Kampkotter, A.; Chovolou, Y.; Kulawik, A.; Rohrdanz, E.; Weber, N.; Proksch, P.; Watjen, W. Isoflavone daidzein possesses no antioxidant activities in cell-free assays but induces the antioxidant enzyme catalase. *Nutr. Res. (N. Y., NY, U. S.)* **2008**, *28*, 620−628.
(14) Chen, Y. C.; Inaba, M.; Abe, N.; Hirota, A. Antimutagenic activity of 8-hydroxyisoflavones and 6-hydroxydaidzein from soybean miso. *Biosci. Biotechnol. Biochem.* **2003**, *67*, 903−906.
(15) Chang, T. S.; Ding, H. Y.; Lin, H. C. Identifying 6,7,4′-trihydroxyisoflavone as a potent tyrosinase inhibitor. *Biosci. Biotechnol. Biochem.* **2005**, *69*, 1999−2001.
(16) Mueller, M.; Jungbauer, A. Red clover extract: a putative source for simultaneous treatment of menopausal disorders and the metabolic syndrome. *Menopause* **2008**, *15*, 1120−1131.
(17) Choi, S. S.; Cha, B. Y.; Lee, Y. S.; Yonezawa, T.; Teruya, T.; Nagai, K.; Woo, J. T. Magnolol enhances adipocyte differentiation and glucose uptake in 3T3-L1 cells. *Life Sci.* **2009**, *84*, 908−914.
(18) Zhang, W. Y.; Lee, J. J.; Kim, I. S.; Kim, Y.; Myung, C. S. Stimulation of glucose uptake and improvement of insulin resistance by aromadendrin. *Pharmacology* **2011**, *88*, 266−274.
(19) Manaharan, T.; Ming, C. H.; Palanisamy, U. D. Syzygium aqueum leaf extract and its bioactive compounds enhances pre-adipocyte differentiation and 2-NBDG uptake in 3T3-L1 cells. *Food Chem.* **2013**, *136*, 354−363.
(20) Nugent, C.; Prins, J. B.; Whitehead, J. P.; Savage, D.; Wentworth, J. M.; Chatterjee, V. K.; O'Rahilly, S. Potentiation of glucose uptake in 3T3-L1 adipocytes by PPAR gamma agonists is maintained in cells expressing a PPAR gamma dominant-negative mutant: evidence for selectivity in the downstream responses to PPAR gamma activation. *Mol. Endocrinol.* **2001**, *15*, 1729−1738.
(21) Chang, L.; Chiang, S. H.; Saltiel, A. R. Insulin signaling and the regulation of glucose transport. *Mol. Med.* **2004**, *10*, 65−71.
(22) Wu, Z.; Xie, Y.; Morrison, R. F.; Bucher, N. L.; Farmer, S. R. PPARgamma induces the insulin-dependent glucose transporter GLUT4 in the absence of C/EBPalpha during the conversion of 3T3 fibroblasts into adipocytes. *J. Clin. Invest.* **1998**, *101*, 22−32.
(23) Kwon, D. Y.; Hong, S. M.; Lee, J. E.; Sung, S. R.; Park, S. Long-term consumption of fermented soybean-derived Chungkookjang attenuates hepatic insulin resistance in 90% pancreatectomized diabetic rats. *Horm. Metab. Res.* **2007**, *39*, 752−757.
(24) Jayagopal, V.; Albertazzi, P.; Kilpatrick, E. S.; Howarth, E. M.; Jennings, P. E.; Hepburn, D. A.; Atkin, S. L. Beneficial effects of soy phytoestrogen intake in postmenopausal women with type 2 diabetes. *Diabetes Care* **2002**, *25*, 1709−1714.
(25) Gyoergy, P.; Murata, K.; Ikehata, H. Antioxidants isolated from fermented soybeans (tempeh). *Nature* **1964**, *203*, 870−872.
(26) Klus, K.; Barz, W. Formation of polyhydroxylated isoflavones from the soybean seed isoflavones daidzein and glycitein by bacteria isolated from tempe. *Arch. Microbiol.* **1995**, *164*, 428−434.
(27) Esaki, H.; Kawakishi, S.; Morimitsu, Y.; Osawa, T. New potent antioxidative o-dihydroxyisoflavones in fermented Japanese soybean products. *Biosci. Biotechnol. Biochem.* **1999**, *63*, 1637−1639.
(28) Seo, S. G.; Yang, H.; Shin, S. H.; Min, S.; Kim, Y. A.; Yu, J. G.; Lee, D. E.; Chung, M. Y.; Heo, Y. S.; Kwon, J. Y.; Yue, S.; Kim, K. H.; Cheng, J. X.; Lee, K. W.; Lee, H. J. A metabolite of daidzein, 6,7,4′-trihydroxyisoflavone, suppresses adipogenesis in 3T3-L1 preadipocytes via ATP-competitive inhibition of PI3K. *Mol. Nutr. Food Res.* **2013**, *57*, 1446−1455.
(29) Wu, Z.; Rosen, E. D.; Brun, R.; Hauser, S.; Adelmant, G.; Troy, A. E.; McKeon, C.; Darlington, G. J.; Spiegelman, B. M. Cross-regulation of C/EBP alpha and PPAR gamma controls the transcriptional pathway of adipogenesis and insulin sensitivity. *Mol. Cell* **1999**, *3*, 151−158.