Cirsium japonicum Flavones Enhance Adipocyte Differentiation and Glucose Uptake in 3T3-L1 Cells

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Cirsium japonicum flavones have been demonstrated to possess anti-diabetic effects in diabetic rats, but the functional mechanism remains unknown. The nuclear receptor peroxisome proliferator-activated receptor γ (PPARγ) plays an important role in glucose and lipid homeostasis. In this study, we report the effects of Cirsium japonicum flavones (pectolinarin and 5,7-dihydroxy-6,4-dimethoxy flavone) on PPARγ activation, adipocyte differentiation, and glucose uptake in 3T3-L1 cells. Reporter gene assays and Oil Red O staining showed that Cirsium japonicum flavones induced PPARγ activation and enhanced adipocyte differentiation of 3T3-L1 cells in a dose-dependent manner. In addition, Cirsium japonicum flavones increased the expression of PPARγ target genes, such as adiponectin and glucose transporter 4 (GLUT4), and enhanced the translocation of intracellular GLUT4 to the plasma membrane. In mature 3T3-L1 adipocytes, Cirsium japonicum flavones significantly enhanced the basal and insulin-stimulated glucose uptake. The flavones-induced effects in 3T3-L1 cells were abolished by the PPARγ antagonist, GW9662, and by the phosphatidylinositol 3-kinase (PI3K) inhibitor, wortmannin. This study suggests that Cirsium japonicum flavones promote adipocyte differentiation and glucose uptake by inducing PPARγ activation and then modulating the insulin signaling pathway in some way, which could benefit diabetes patients.

Key words Cirsium japonicum; flavone; peroxisome proliferator-activated receptor γ; adipocyte differentiation; adiponectin; glucose transporter 4

Adipocytes play a major role in energy homeostasis. Excess numbers of adipocytes or enlarged adipocyte size is the hallmark of obesity, which is a major risk factor for the development of type II diabetes (T2DM).1,2) Strict regulation of adipocyte differentiation and adipogenesis is essential for preventing obesity. Peroxisome proliferator-activated receptor γ (PPARγ) is one of the most important regulators of adipocyte differentiation,3) and is predominantly expressed in adipose tissue as a transcription factor belonging to a sub-family of nuclear hormone receptors.4) Studies have shown that PPARγ enhances insulin sensitivity by increasing the numbers of insulin-sensitive adipocytes,5) or by regulating the expression of a number of genes such as adiponectin and glucose transporter 4 (GLUT4), which are involved in the regulation of the insulin signaling pathway in adipocytes.5) Both adiponectin and GLUT4 mediate glucose uptake and lipid metabolism in insulin-responsive tissues such as adipose tissue, which is essential for the maintenance of whole-body glucose homeostasis.7) The role of PPARγ in the development and treatment of diabetes has been well established by several research groups.8–10) Loss-of-function mutations in the PPARγ gene caused severe lipodystrophy and diabetes, indicating the importance of PPARγ in metabolism.11,12) Thus, PPARγ has been identified as an appropriate target for pharmacological interventions for treating diabetes.13)

More and more compounds isolated from natural plants have been reported to activate PPARγ thereby enhancing insulin sensitivity and promoting adipocyte differentiation.14–18) Cirsium japonicum is a wild perennial herb that has been widely used as an antihemorrhagic and diuretic agent. Flavonoid compounds are the major chemical content in Cirsium japonicum. It has been reported that Cirsium japonicum flavones exert antimicrobial, antioxidant and antitumor effects.19,20) While scientists reported that the methanol and water extracts from Cirsium japonicum roots possess antioxidant and anti-diabetic effects by inhibiting alpha-glucosidase activity,21) our lab demonstrated in diabetic rats the anti-diabetic effect of two flavones, pectolinarin and 5,7-dihydroxy-6,4-dimethoxy flavone (DDMF), isolated from Cirsium japonicum.22) However, the functional mechanism remains unknown. In the present study, we investigated the effect of Cirsium japonicum flavones on adipocyte differentiation and glucose uptake in 3T3-L1 cells. Our research revealed that Cirsium japonicum flavones stimulated adipocyte differentiation and enhanced glucose transport in adipocytes by inducing the PPARγ-mediated expression of adiponectin and translocation of GLUT4 vesicles in 3T3-L1 adipocytes.

MATERIALS AND METHODS

Materials PPARγ-luciferase reporter plasmid, fetel bovine serum (FBS), Dulbecco’s modified Eagle’s medium (DMEM), penicillin and streptomycin were from Invitrogen. Dexamethasone (DEX), 3-isobutyl-methylxanthine (IBMX), insulin, GW9662, Wortmannin, Oil Red O and [2-3H]deoxyglucose were from Sigma (St. Louis, MO, U.S.A). Rosiglitazone was purchased from Alexis Biochemicals (San Diego, CA, U.S.A.). Polyclonal antibodies against GLUT4, adiponectin, glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and horse-radish peroxidase (HRP)-linked anti-rabbit immunoglobulin G (IgG) were from Cell Signaling Technology Inc. (Beverly, MA, U.S.A.). Trizol reagent, Qiagen RNasey MinElute cleanup kit, QuantiTect SYBR Green reverse transcription-polymerase chain reaction (RT-PCR) Kit and luciferase reporter assay systems were from Promega (San Luis Obispo, CA, U.S.A.). Pierce® bicinchoninic acid (BCA) Protein Assay kit and enhanced chemiluminescence reagent were from Pierce Biotechnology, U.S.A. FuGENE® 6 transfection reagent was from
Roche (Mannheim, Germany). Pectolinarin and DDMF were previously prepared in our lab\textsuperscript{22} (structure shown in Fig. 1).

**Cell Culture and Reporter Gene Assay** Mouse 3T3-L1 preadipocytes were obtained from Cell Resources Center, Shanghai Institutes for Biological Sciences, Chinese Academy of Sciences, and cultured in DMEM supplemented with 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin at 37°C in a humidified 5% CO\textsubscript{2} atmosphere. 3T3-L1 preadipocytes grown in 24-well plates were transfected with a PPAR\textgamma-luciferase reporter plasmid and β-gal plasmid using FuGENE\textsuperscript{®} 6 transfection reagent. Twenty-four hours after transfection, cells were treated with or without *Cirsium japonicum* flavonoids for another 24 h. Cells were treated with or without DEX, insulin (1.0 µg/mL) or GW9662. Measurement of intracellular lipid accumulation using Oil Red O staining was then performed to monitor the degree of increase compared with basal 3T3-L1 cells and normalized against protein concentration in each sample.

**Quantitative RT-PCR** Total RNA was extracted from cells with Trizol reagent. After RNase-free DNase I digestion, mRNA was prepared by purifying total RNA with Qiagen RNeasy MinElute cleanup kit. The purified mRNA was then reverse-transcribed to cDNA using random primers and a reverse transcription system. Quantitative real-time PCR was performed with QuantiTect SYBR Green RT-PCR Kit and the fluorescence values were converted into threshold cycle values using ABI PRISM\textsuperscript{®} 7000 Sequence Detector Program according to the manufacturer’s protocol. PCR amplifications were carried out as follows: 1 cycle for 10 min at 94°C, followed by 35 cycles of 94°C for 15 s, 62°C for 30 and 72°C for 45 s. β-Actin mRNA was used as an internal control to normalize the determined mRNA levels. Primers were designed as follows: adiponectin, 5'-GTGCAAGGGTGGATGCGAGG-3' and 5'-ATGTTCTGTGTTGTTAGGAG-3'; GLUT4, 5'-GTG ACTGGAAACCTGTCTAG-3' and 5'-GAAAGGAGAGATAGGACAC-3'; β-actin, 5'-AGAAGATTTTGCCACCCAATTC-3' and 5'-GAGGGATCTGTCAGGGTCCC-3'. All assays were performed in triplicate.

**Preparation of Cell Extracts and Western Blot Analysis** Cells were washed with ice-cold PBS, scraped, collected, lysed in ice-cold buffer I and centrifuged at 15000×*g* for 20 min at 4°C. Supernatants were saved and stored at −80°C. For subcellular fractionation, cells were lysed in buffer III (0.25 M sucrose, 2 mM EDTA, 1 mM phenylmethylsulfonyl 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 12000×*g* for 20 min to isolate the crude plasma membrane fraction as the pellets. The supernatants were collected as the cytosolic fraction and the crude plasma membrane pellets were re-suspended in buffer III. Protein concentrations were measured using the Pierce\textsuperscript{®} BCA Protein Assay kit. Samples with equal amount of protein were denatured, subjected to 10% SDS-PAGE and transferred to nitrocellulose membranes. Membranes were incubated overnight at 4°C with the rabbit anti-GLUT4, adiponectin or GAPDH antibodies in PBS containing 5% (w/v) BSA. Membranes were washed three times with Tris–HCl, pH 7.4, and lysed with ice-cold buffer II (118 mM NaCl, 4.7 mM KCl, 1.0 mM CaCl\textsubscript{2}, 1.0 mM MgSO\textsubscript{4}, 25 mM Na\textsubscript{2}HPO\textsubscript{4}, 1.2 mM K\textsubscript{2}HPO\textsubscript{4}, 0.5% BSA, 20 mM N-(2-hydroxyethyl)piperazine-N’-2-ethanesulfonic acid (Hepes), 2 mM sodium pyruvate, 0.1 mM [2-3H]deoxyglucose, pH 7.4) supplemented with the indicated concentrations of *Cirsium japonicum* flavones in the presence or absence of 0.1 µM insulin for 30 min. After three washes with cold [2-3H]-deoxyglucose-free buffer II, cells were solubilized in 0.1% sodium dodecyl sulfate (SDS) and the radioactivity was determined in a scintillation counter. Glucose uptake was expressed as the amounts of [2-3H]-deoxyglucose uptake and the uptake was normalized against protein concentration in each sample.
times and incubated with the goat anti-rabbit IgG conjugated to HRP in PBS containing 5% (w/v) BSA. The blots were developed by enhanced chemiluminescence.

**Statistical Analysis** All quantitative data are representative of at least three independent experiments, and expressed as means±S.E. The statistical significance of differences between the data pairs was evaluated by analysis of variance (ANOVA).

**RESULTS**

**Cirsium japonicum Flavones Activate PPARγ** It has been reported that *Cirsium japonicum* flavones showed an antidiabetic effect in diabetic rats. To study whether the *Cirsium japonicum* flavones-improved adiponectin expression and glucose metabolism in diabetic rats is related to the PPARγ pathway, we explored the effects of pectolinarin and DDMF on PPARγ activity in 3T3-L1 preadipocytes. Preadipocytes transfected with PPARγ-luciferase reporter gene were treated with pectolinarin or DDMF at the indicated concentrations in the absence or presence of the PPARγ antagonist, GW9662. The PPARγ agonist, rosiglitazone, was used here as a positive control. Treatment of preadipocytes with pectolinarin or DDMF significantly activated PPARγ in a dose-dependent manner, which was inhibited by co-treatment with the PPARγ specific antagonist, GW9662 (Fig. 2A). Moreover, both quantitative RT-PCR analysis and western blot data showed that the expression of PPARγ target genes such as adiponectin and GLUT4 increased accordingly in pectolinarin or DDMF-treated adipocytes (Figs. 2B–D), which was also inhibited by GW9662. Because some compounds from other natural plants have been reported to be the ligand of PPARγ, we tried to determine the affinity of pectolinarin and DDMF for PPARγ. However, there was no detectable binding of pectolinarin and DDMF to PPARγ (data not shown here). These findings indicate that *Cirsium japonicum* flavones are positive activators of PPARγ, and possibly function upstream of PPARγ.

**Cirsium japonicum Flavones Promote Adipocyte Differentiation of 3T3-L1 Cells** It is known that PPARγ activation is a key process during adipocyte differentiation. Based on this, a number of compounds from natural plants are being developed for pharmacological interventions for obesity-related diabetes treatment. Because *Cirsium japonicum* flavones induced PPARγ activation, especially up-regulating the expression of the PPARγ target gene adiponectin, which is involved in the induction of adipocyte differentiation and lipid metabolism, we investigated the effects of pectolinarin and DDMF on adipocyte differentiation of 3T3-L1 cells. As shown in Fig. 3, both pectolinarin and DDMF induced adipocyte differentiation of 3T3-L1 cells at a dose-dependent manner. The optical densities of the Oil Red O-stained extractions from cells treated with pectolinarin at concentrations of 1.0 μM, 10 μM, and 50 μM increased by 22%, 78% and 98%, respectively (p<0.05). Treatment of 3T3-L1 preadipocytes with 1.0 μM, 10 μM and 50 μM DDMF resulted in 17%, 61% and 87% increase in the optical densities of the Oil Red O-stained extractions, respectively (p<0.05). The positive control, PPARγ agonist rosiglitazone (1.0 μM), also significantly promoted adipocyte differentiation of 3T3-L1 preadipocytes (p<0.05). To further confirm the effect of *Cirsium japonicum* flavones as a PPARγ activator and to elucidate at which level in the PPARγ signaling pathway this activation occurs, we examined the effects of the PPARγ antagonist, GW9662, on *Cirsium japonicum* flavones-induced differentiation. As shown in Fig. 3, the lipid accumulation in 3T3-L1 preadipocytes treated with *Cirsium japonicum* flavones, insulin and GW9662 was greatly reduced, compared with the 3T3-L1 preadipocytes that were not treated with GW9662. Taken together, these results indicate that *Cirsium japonicum* flavones enhance adipocyte differentiation via inducing PPARγ activation.

**Cirsium japonicum Flavones Increase Basal and Insulin-Stimulated Glucose Uptake in 3T3-L1 Adipocytes** It has been reported that PPARγ activation plays an important role in glucose metabolism, especially in enhancing glucose uptake in 3T3-L1 adipocytes. Next, we investigated the effects of *Cirsium japonicum* flavones on glucose metabolism and insulin sensitivity in 3T3-L1 adipocytes. As shown in Fig. 4, *Cirsium japonicum* flavones enhanced both basal and insulin-stimulated glucose consumption in a dose-dependent...
manner in 3T3-L1 adipocytes. Treatment with pectolinarin at concentrations of 1.0 \( \mu M \), 10 \( \mu M \) and 50 \( \mu M \) increased the basal glucose uptake by 31%, 56%, and 67% \((p<0.05)\), respectively, compared with the vehicle-treated cells. The basal glucose uptake in 1.0 \( \mu M \), 10 \( \mu M \) and 50 \( \mu M \) DDMF-treated cells increased by 25%, 45%, and 60% \((p<0.05)\), respectively. The insulin-stimulated glucose uptake increased by 13%, 75% and 131% \((p<0.01)\) in pectolinarin-treated cells at concentrations of 1.0 \( \mu M \), 10 \( \mu M \) and 50 \( \mu M \), respectively, and by 23%, 77% and 125% \((p<0.01)\) in DDMF-treated cells at concentrations of 1.0 \( \mu M \), 10 \( \mu M \) and 50 \( \mu M \), respectively. These effects of pectolinarin and DDMF were significantly inhibited by GW9662. In addition, the GLUT4 level in the plasma membrane increased after treatment with pectolinarin or DDMF. Because activation of phosphatidylinositol 3-kinase (PI3K) is necessary for glucose transport and GLUT4 translocation,25) we examined the effects of wortmannin on \textit{Cirsium japonicum} flavones-enhanced glucose uptake. The results show that 0.1 \( \mu M \) wortmannin significantly inhibited both pectolinarin and DDMF-stimulated glucose uptake and GLUT4 translocation (Fig. 4). These results indicate that \textit{Cirsium japonicum} flavones increase both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes, possibly through modulating the insulin signaling pathway.

**DISCUSSION**

Diabetes mellitus is considered to be a serious endocrine syndrome. Obesity is a risk factor for diabetes and hyperlipidemia, and the exploding prevalence of obesity-induced insulin resistance and T2DM has become a major public health problem worldwide. Adipose tissue has been identified as an endocrine organ, which secretes a series of bioactive molecules named adipocytokines, involved in maintaining metabolic homeostasis.26) Adipose tissue functions in insulin resistance syndrome through the production and secretion of adipocytokines, such as aP2, adiponectin, GLUT4, GLUT1, PPAR\(\gamma\), plasminogen activator inhibitor-1 (PAI-1) and tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)), which are differentially expressed and regulated in enlarged adipocytes and small adipocytes.26,27) Because dysregulation of the number of adipocytes greatly contributes to the development of obesity-related T2DM, positive regulation of adipocyte differentiation and adipogenesis is of great significance for preventing obesity-induced insulin resistance and T2DM. Conversion of preadipocytes to adipocytes is controlled by various regulators, especially PPAR\(\gamma\) which has been clinically validated for therapeutic interventions in treating T2DM.28) More and more studies have shown that natural compounds work well in improving hyperglycemia and hypertriglyceridemia, most likely by remodeling the adipose tissue, whereby the number of large adipocytes decreases.
positively regulate glucose uptake in adipocytes. Adiponectin is an adipose tissue-derived hormone of the C1q/TNF-superfamily, which is highly abundant in human serum and shows an inverse correlation with body mass index. Higher adiponectin levels are associated with a lower risk for type 2 diabetes, and the plasma adiponectin concentration is inversely correlated with the severity of insulin resistance. Full-length and globular adiponectin promote the translocation of GLUT4 to the plasma membrane. 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. PPARγ activators are known to increase glucose transport in adipocytes by directly regulating the expression of PPARγ target genes such as adiponectin and GLUT4, which are involved in glucose metabolism. In accordance with the finding that Cirsium japonicum flavones activated PPARγ, in Cirsium japonicum flavones-treated adipocytes the expression of adiponectin and GLUT4 and GLUT4 translocation to the plasma membrane were up-regulated. As a result, Cirsium japonicum flavones increased both basal and insulin-stimulated glucose uptake, with maximal stimulation at 50 μM for both pectolinarin and DDMF. It is known that activation of PI3K is a major event in the insulin signaling cascade that leads to GLUT4 translocation. Binding of insulin to insulin receptor (IR) results in autophosphorylation of the intracellular tyrosine residues of the IR β-subunit, which leads to the activation of downstream signaling molecules such as PI3K and GLUT4 translocation to the plasma membrane. Here, we found that Cirsium japonicum flavones-increased basal and insulin-stimulated glucose uptake was blocked by the PI3K inhibitor, wortmannin. At the same time, the ratio of plasma membrane GLUT4 to total GLUT4 increased after Cirsium japonicum flavones treatment, which was inhibited by wortmannin. These results suggest that Cirsium japonicum flavones may modulate the insulin signaling pathway, thereby positively regulating glucose uptake in adipocytes. However, further studies are required to clarify whether there are alternative pathways responsible for the Cirsium japonicum flavones-induced effects.

In summary, we found that Cirsium japonicum flavones enhanced adipocyte differentiation by increasing the PPARγ transcriptional activity. In addition, Cirsium japonicum flavones promoted both basal and insulin-stimulated glucose uptake in 3T3-L1 adipocytes, possibly through increased adiponectin and GLUT4 expression and GLUT4 translocation, which was at least partially related to the modulation of insulin signaling. Our research further demonstrates that Cirsium japonicum flavones have the potential of being used as an active pharmaceutical ingredient for treating diabetes.

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