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Luteolin enhances insulin sensitivity via activation of PPARγ transcriptional activity in adipocytes

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Abstract

Obesity and insulin resistance have been linked to a low-grade chronic inflammatory response characterized by increased macrophage infiltration, altered cytokine production and activation of inflammatory signaling pathway in adipose tissue. Pharmacological agents and natural products that are capable of reducing inflammatory activity possess anti-diabetic properties. Luteolin, a naturally occurring flavonoid, has been demonstrated to inhibit lipopolysaccharide-induced tumor necrosis factor-α (TNFα) release and activation of NF-κB pathway in macrophages. However, little is known about the mechanism and effect of luteolin on inflammation-related insulin resistance in adipocytes. In this study, we investigated the effect of luteolin on insulin action in 3T3-L1 adipocytes and primary adipose cells. Here we showed that luteolin treatment for 24 h increased the response of glucose uptake to insulin stimulation in 3T3-L1 adipocytes. Our results also demonstrated that luteolin enhanced Akt2 phosphorylation in an insulin-stimulated state. Furthermore, luteolin treatment decreased mRNA levels of TNFα, interleukin-6 and MCP-1, while it increased the gene expression of adiponectin and leptin in 3T3-L1 adipocytes and primary mouse adipose cells. Most interestingly, we found that treatment of luteolin markedly enhanced peroxisome proliferator-activated receptor γ (PPARγ) transcriptional activity in 3T3-L1 adipocytes, and luteolin-increased expression of adiponectin and leptin was blocked by GW9662, a PPARγ antagonist. Thus, our data suggest that luteolin influences insulin action and production of adipokines/cytokines in adipocytes by activating the PPARγ pathway.
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Keywords: Flavonoids; Adipocyte; Peroxisome proliferator-activated receptor γ; Insulin sensitivity

1. Introduction

It is well documented that chronic low-grade inflammation characterized by dysregulated cytokine production, increased inflammatory mediators and activation of inflammatory signaling pathways is associated with obesity, insulin resistance and type 2 diabetes [1]. Adipose tissue is now recognized as an endocrine organ, in addition to the traditional view of adipose tissue being an organ for energy storage and a major site of increased macrophage infiltration and inflammatory response in obesity. The main identified adipokines/cytokines secreted by adipose tissue include adiponectin, leptin, tumor necrosis factor-α (TNFα), interleukin-6 (IL-6) and CC chemokine ligand 2 (also known as MCP-1). These adipokines/cytokines have been demonstrated to play important roles in the regulation of insulin action and metabolism.

In obesity and insulin resistance, secretion of adipokines and cytokines is dysregulated. For example, serum levels of adiponectin, a secreted molecule with insulin-sensitizing effect, are significantly decreased in rodents and humans with obesity, insulin resistance and type 2 diabetes mellitus [2], while increased expression and/or secretion of cytokines, including TNFα and IL-6, has been observed in adipose tissues of obese and insulin-resistant rodents and humans [3]. Inflammatory cytokines, especially TNFα, reduce insulin action by activating the major proinflammatory IKKβ/NF-κB pathway, leading to inhibition of insulin signal transduction and insulin resistance [4,5] such as down-regulation of the expression of IRS-1 and glucose transporter (GLUT) 4 and reduction in insulin-stimulated glucose uptake [6].

Peroxisome proliferator-activated receptor γ (PPARγ) is a ligand-activated transcription factor belonging to the nuclear receptor superfamily that is crucial for the regulation of adipogenesis, lipid metabolism and glucose homeostasis [7]. Besides, PPARγ has also been demonstrated to be a negative regulator of obesity-associated inflammatory responses and a key activator of insulin sensitivity [7]. PPARγ and NF-κB mutually antagonize each other's activities by a mechanism involving transcriptional repression. For instance, studies have shown that PPARγ inhibits the expression of proinflammatory genes by interfering with signal-dependent activation of the NF-κB pathway [8]. On the other hand, NF-κB activation attenuates PPARγ activity, leading to insulin resistance in the cell. Apparently, PPARγ is a good therapeutic target for insulin resistance, and the TZD class of PPARγ ligands has been developed for the treatment of type 2 diabetes.

In recent years, nutriceutical interventions that could improve insulin resistance have been considered as a complementary approach to treat type 2 diabetes. Among bioactive compounds that have been tested for potential beneficial health effects, flavonoids are one of the groups of naturally occurring compounds with anti-

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inflammatory [9] and anti-cancer properties [10]. Luteolin (3′,4′,5,7-tetrahydroxyflavone), one member of the flavonoid group isolated from *L. japoinica*, is present at high concentrations in celery, green pepper, perilla leaf and seeds, and chamomile [11]. Evidence from previous *in vitro* studies has suggested that luteolin is one of the most potent and most efficacious flavonoids that have anti-inflammatory activity. More specifically, luteolin inhibits the lipopolysaccharide (LPS)-induced production of TNFα, IL-6 and inducible nitric oxide in macrophages [12,13], as well as suppresses TNFα-induced NF-κB pathway activation in cancer cells [14].

Herein we investigated the potential anti-insulin-resistance properties of luteolin by examining the effect of luteolin on insulin action in adipocytes. Our results demonstrated that luteolin increases insulin action, decreases gene expression of inflammatory cytokines and induces gene and protein expression of PPARγ_2 and GLUT4, adiponectin and leptin in a PPARγ-activation-dependent manner.

2. Materials and methods

2.1. 3T3-L1 cell culture

3T3-L1 cells were grown at 37°C and 5% CO_2 in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen, Carlsbad, CA) with 100 IU/ml penicillin/streptomycin (Invitrogen) and 10% bovine calf serum (BCS; Sigma Aldrich, St. Louis, MO) until confluent and induced to differentiate into adipocytes [15]. On Day 8 of differentiation, adipocytes were exposed to 0.5% BCS and 1 mg/ml glucose for 2 h, followed by various treatments: (a) control; (b) luteolin (20 μmol/L) for 6 h and insulin for an additional 30 min; (c) luteolin (20 μmol/L) for 24 h; and (d) GW9662 (Sigma Aldrich) (10 μmol/L) for 3 h and luteolin for an additional 24 h. At the end of the experiments, cells were prepared for [^{3}H]2-deoxy-D-glucose uptake, RNA extraction by quantitative real-time RT-PCR assay and protein collection by Western blot analysis.

2.2. Primary mouse adipose cell isolation and culture

Preparation of isolated mouse epididymal adipose cells from four C57BL/6J mice was performed as described previously [16,17]. After fat pads had been minced, they were digested with collagenase (2 mg/ml solution) in digestion vials containing Krebs–Ringer bicarbonate Hepes buffer (pH 7.4), 200 nM adenosine (KRBH buffer) and 3.5% bovine serum albumin (BSA). After a 2-h digestion, adipose cells and stromal–vascular cells were separated by centrifugation at 1200 rpm for 10 min. The isolated adipose cells were washed twice with KRBH buffer containing 3.5% BSA and washed twice with 3.5% BSA DMEM by centrifugation at 1000 rpm for 2 min. After the final wash, adipose cells were cultured in 3.5% BSA DMEM in the presence or in the absence of luteolin (20 μmol/L) at 37°C for 6 h in 5% CO_2. After incubation, the cells were collected and prepared for total RNA extraction.

2.3. Glucose uptake assay

Uptake of 2-deoxy-D-[^{3}H]glucose (Amersham Biosciences, Piscataway, NJ) was measured in 3T3-L1 adipocytes, as previously described [18]. Briefly, cells were serum starved in KRH buffer supplemented with 0.5 % BSA and 2 mmol/L sodium pyruvate (pH 7.4) for 3 h and then incubated either with or without 173 nmol/L insulin for 30 min at 37°C. Glucose uptake was initiated by the addition of [^{3}H]2-deoxy-D-glucose to a final assay concentration of 100 μmol/L at 37°C. After 5 min, 2-deoxyglucose uptake was terminated by three washes with ice-cold KRH buffer, and the cells were solubilized with 0.8 ml of KRH buffer containing 1% Triton X-100. Incorporated radioactivity was determined by scintillation counting. Nonspecific 2-deoxyglucose uptake was measured in the presence of 20 μmol/L cytocholasin B and subtracted from the total glucose uptake assayed to obtain specific uptake.

2.4. Western blot analysis

Total proteins of 3T3-L1 adipocytes were extracted in a solubilization buffer containing 25 mmol/L Tris–HCl (pH 7.5), 0.5 mmol/L EDTA, 25 mmol/L sodium chloride, 10 mmol/L sodium fluoride, 1 mmol/L sodium vanadate, 1% Nonidet P-40 and protease inhibitor cocktails (Diagnostic Roche, Branchberg, NJ). Nuclear proteins of 3T3-L1 adipocytes were extracted in accordance with the manufacturer's instructions (Active Motif, Carlsbad, CA). Briefly, 3T3-L1 cells were harvested and incubated with hypotonic buffer for 15 min on ice, followed by detergent treatment for 10 s at the highest vortex setting, then cytosol proteins in supernatants were collected after centrifugation at 14,000×*g*. Nuclear pellets were then resuspended in lysis buffer, and nuclear proteins were acquired by centrifugation at 14,000×*g* for 10 min. Protein concentrations of lysates were detected with the bicinchoninic acid method (Pierce, Rockford, IL). Equivalent proteins (70 μg of total proteins) were separated on SDS-PAGE and immunoblotted with anti-Akt2-phospho Ser473 and anti-Akt2 (Cell Signaling Technology, Inc., Danvers, MA), anti-PPARγ (Cell Signaling Technology, Inc.), anti-β-actin (Sigma Aldrich) and anti-GLUT4 (kindly provided by Dr. Samuel Cushman, NIDDK/National Institutes of Health) antibodies, in accordance with the recommendations of the manufacturers. Following incubation with primary antibodies, the membranes were incubated with secondary antibodies conjugated to horseradish peroxidase. Antibody reactivity was detected by ECL Western Blotting Detection Systems (GE Healthcare Bio-Sciences Corp., Piscataway, NJ).

2.5. Electrophoretic mobility shift assay (EMSA)

Nuclear extract was prepared from Day-8-differentiated 3T3-L1 adipocytes treated with or without 20 μM luteolin for 6 and 12 h using Nuclear Extract kit (Active Motif). Two double-stranded deoxyoligonucleotides (5′-CCCTCGACCCTACCTTTGTCC-CACTCCTGC-3′) containing the PPRE of the mouse HSL promoter were 5′ end labeled with biotin (Invitrogen). Nonradioactive EMSA was detected according to a kit manual (cat no. 20148; Pierce). Nuclear extracts (1.5 μg of protein) were incubated in 20 μl of binding buffer [10 mM Tris–HCl (pH 7.5), 50 mM $MgCl_2$ and 0.05% Nonidet P-40] containing 1 μg of poly(dI–dC) and 20 fmol of biotin-labeled double-stranded DNA probe for 20 min at room temperature. Samples were separated by electrophoresis in 6% nondenaturing polyacrylamide gels and transferred to nylon membranes. The membranes were blocked with 5% BSA and incubated with streptavidin-conjugated horseradish peroxidase. After the membranes had been adequately washed, they were then visualized with chemiluminescence solution, followed by exposure to X-ray film. The specificity of the probes was evaluated by incubating the mixed nuclear extracts from all groups of samples with excess (200×) unlabeled double-stranded probes.

2.6. Relative quantitative real-time RT-PCR

Total RNAs were extracted from 3T3-L1 adipocytes and primary mouse adipose cells with various treatments using TRIzol reagent (Invitrogen), in accordance with the manufacturer's instructions. RNA was treated with RQ1 DNase (Promega, Madison, WI) at 37°C for 30 min. First-strand cDNA was generated using the oligo(dT) primer (Promega), and 10 μl of diluted cDNA (1:20) was used in each 25-μl real-time PCR using the SYBR GreenER qPCR SuperMix Universal kit (Invitrogen) with an ABI 7500 Real-Time PCR System (Applied Biosystems, Foster City, CA). Results were analyzed using the 7500 system SDS software supplied with the 7500 system. Primers specific for the genes examined are listed in Table 1. Results were presented as levels of expression relative to those of controls after normalization to β-actin using the $\Delta\Delta C_t$ method. Statistical significance was determined by two-tailed Student's *t* test.

2.7. Statistical analysis

Results were expressed as mean±standard error of the mean. Differences in parameters between groups were analyzed using a two-group *t* test or a multipaired *t* test with a .05 two-sided significance level. *P*<.05 was considered significant.

Table 1
Sequences of primers for real-time RT-PCR

Accession no.	Gene	Primer sequence	
		Forward	Reverse
AY754346	*Adiponectin*	5′-GCAGAGATGGCACTCCTGGA-3′	5′-CCCTTCAGCTCCTGTCATTCC-3′
NM_008493	*Leptin*	5′-CCACACACAGCTGGAAACTCC-3′	5′-GGCTTGCTTCAGATCCATCC-3′
NM_011146	*PPARγ_2*	5′-TTGACCCAGAGCATGGTGC-3′	5′-GAAGTTGGTGGGCCAGAATG-3′
NM_011333	*MCP-1*	5′-CTTCTGGGCCTGCTGTTCA-3′	5′-GAGTAGCAGCAGGTGAGTGGG-3′
NM_013693	*TNFα*	5′-GAGTAGCAGCAGGTGAGTGGG-3′	5′-CTGCCACAAGCAGGAATGAG-3′
DQ788722	*IL-6*	5′-AGAAGGAGTGGCTAAGGACCAA-3′	5′-AACGCACTAGGTTTGCCGAG-3′
NM_011400	*GLUT1*	5′-TCAACGAGCATCTTCGAGAAGGCA-3′	5′-TCGTCCAGCTCGCTCTACAACAAA-3′
NM_009204	*GLUT4*	5′-AACGGATAGGGAGCAGAAACCCAA-3′	5′-GTGCAAAGGGTGAGTGAGGCATTT-3′
NM_007393.2	*β-Actin*	5′-CCTAAGGCCAACCGTGAAAA-3′	5′-GAGGCATACAGGGACAGCACA-3′

3. Results

3.1. Luteolin increases insulin-stimulated glucose uptake and insulin signaling activity in 3T3-L1 adipocytes

In order to test the effect of luteolin on glucose metabolism and insulin action in adipocytes, we examined insulin-stimulated glucose transport activity in 3T3-L1 adipocytes. Several studies have shown that the responses of insulin signaling and glucose uptake activity in 3T3-L1 adipocytes do not always occur concurrently. For example, short-term treatment (6 h) of TNFα could significantly induce insulin signaling resistance, while TNFα-induced reduction in glucose uptake activity required longer treatment periods [6,19]. Based on these previous observations, we treated 3T3-L1 adipocytes with luteolin for 24 h to examine its effect on glucose uptake, and for 6 h to test its effect on insulin signaling activity. Day-8-differentiated adipocytes were treated with luteolin at concentrations of 0, 0.1, 1, 10 and 20 μmol/L for 24 h. As illustrated in Fig. 1, the result of 2-deoxy-D-[^{3}H]glucose uptake assay showed that control cells displayed a sevenfold increase in glucose uptake in response to insulin. In cells treated with various concentrations of luteolin for 24 h, the response of glucose uptake to insulin stimulation was increased in a dose-dependent manner as compared to control cells, and the difference in insulin-stimulated glucose uptake was significant (P<.05) at the indicated concentrations (Fig. 1). Multiple t tests showed that fold increases were significantly different between 1 μmol/L luteolin-treated and 0.1 μmol/L luteolin-treated groups (P<.05), as well as between 10 μmol/L luteolin-treated and 0.1 μmol/L luteolin-treated groups. The result also demonstrated a trend toward a decrease in the basal levels of glucose uptake in the cells with luteolin treatment.

In order to further determine the effect of luteolin on insulin action, insulin signaling activity was examined by assessing the phosphorylation state of Akt2 in 3T3-L1 adipocytes. Cells were treated with or without 20 μmol/L luteolin for 6 h and then treated

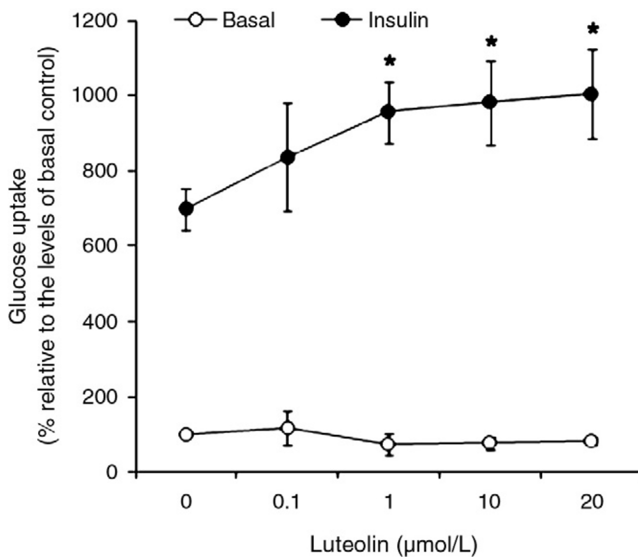

Fig. 1. Luteolin enhances insulin-stimulated glucose uptake in 3T3-L1 adipocytes. Day-8-differentiated 3T3-L1 adipocytes were treated with various doses of luteolin in DMEM containing 0.5% BCS and 1 mg/ml glucose for 24 h. Cells were then serum starved in KRH buffer for 3 h, followed by incubation with [^{3}H]2-deoxy-D-glucose in the absence or in the presence of 100 nmol/L insulin for 30 min, as described in Materials and Methods. Glucose influx was determined. The basal glucose uptake level in control cells was set to 100. The glucose uptake levels in the other treatment conditions were normalized to the basal levels in control cells in each individual experiment. Mean and S.E. were calculated from normalized values from all experiments. The results represent two to four independent experiments (*P<.05, treatment vs. control).

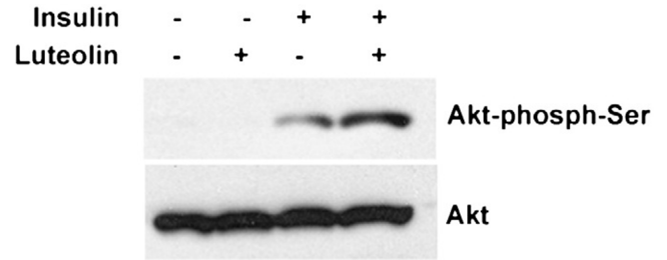

Fig. 2. Luteolin increases Akt serine phosphorylation in 3T3-L1 adipocytes. Day-8-differentiated 3T3-L1 adipocytes were treated with 0.5% BCS and 1 mg/ml glucose for 12 h. Cells were then pretreated with or without 20 μmol/L luteolin for 6 h and treated with insulin for an additional 30 min. Protein levels of Akt2-phospho Ser and total Akt were determined by Western blot analysis. The results represent two independent experiments.

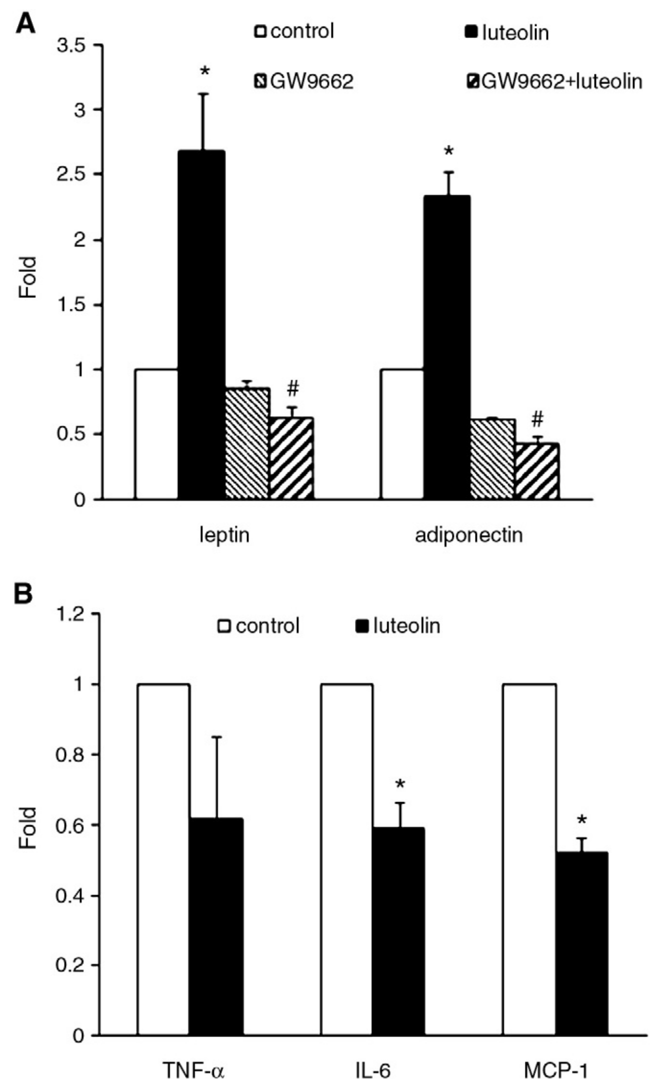

Fig. 3. Luteolin increases the expression levels of adipokines and decreases the expression levels of inflammatory cytokines in 3T3-L1 adipocytes. Day-8-differentiated 3T3-L1 adipocytes were treated with 0.5% BCS and 1 mg/ml glucose for 12 h. Cells were then pretreated with or without GW9662 (a PPARγ antagonist) for 3 h, followed by luteolin (20 μmol/L) for 24 h. Total RNAs were extracted to determine the mRNA levels of leptin and adiponectin (A) and inflammatory cytokines (B). The results are presented as the mean±S.E. of triplicates from three independent experiments. (*P<.05, luteolin treatment vs. control; #P<.05, treatment with luteolin plus GW9662 vs. luteolin alone).

with or without insulin for an additional 30 min. Western blot analysis showed that basal Akt2 phosphorylation was not affected by luteolin treatment. However, in the insulin-stimulated state, Akt2 phosphorylation was largely increased in the cells pretreated with luteolin for 6 h as compared to the cells without luteolin treatment (Fig. 2).

3.2. Luteolin increases the mRNA levels of adipokines and decreases the mRNA levels of inflammatory cytokines in 3T3-L1 adipocytes

As stated earlier, the secretory activities of adipocytes and their secreted products (adipokines/cytokines) play a central role in regulating insulin sensitivity and metabolism. Dysregulation of adipokine/cytokine secretion is associated with insulin resistance and obesity; it is likely to be the core defect of insulin resistance. To this end, we addressed the possible effect of luteolin on adipocyte secretory activity as a test of insulin sensitivity in adipocytes. 3T3-L1 adipocytes on Day 8 of differentiation were incubated with 20 μmol/L luteolin for 24 h. As illustrated in Fig. 3A, luteolin treatment markedly increased the levels of gene expression of two key adipokines, adiponectin and leptin, whereas it significantly reduced the mRNA levels of IL-6 and MCP-1. The mRNA levels of TNFα had a decreasing trend with luteolin treatment, but the difference did not reach a statistically significant level (Fig. 3B).

3.3. Luteolin increases PPARγ pathway activation and PPARγ activation-dependent gene expression of adiponectin and leptin in 3T3-L1 adipocytes

PPARγ, a key anti-inflammatory transcription factor, plays a significant role in the transcriptional regulation of metabolic gene expression. *Adiponectin* and *leptin* are the two key PPARγ target genes [20–22]. To elucidate the potential role of luteolin as a PPARγ agonist, we examined the effect of luteolin on the expression of PPARγ and PPARγ target genes (*adiponectin* and *leptin*). Interestingly, luteolin treatment alone for 24 h significantly increased the gene expression of PPARγ_2 (Fig. 4A).

To assess the effect of luteolin on PPARγ signaling, we examined the nuclear translocation of PPARγ by Western blot analysis of nuclear and cytosol proteins extracted from 3T3-L1 adipocytes

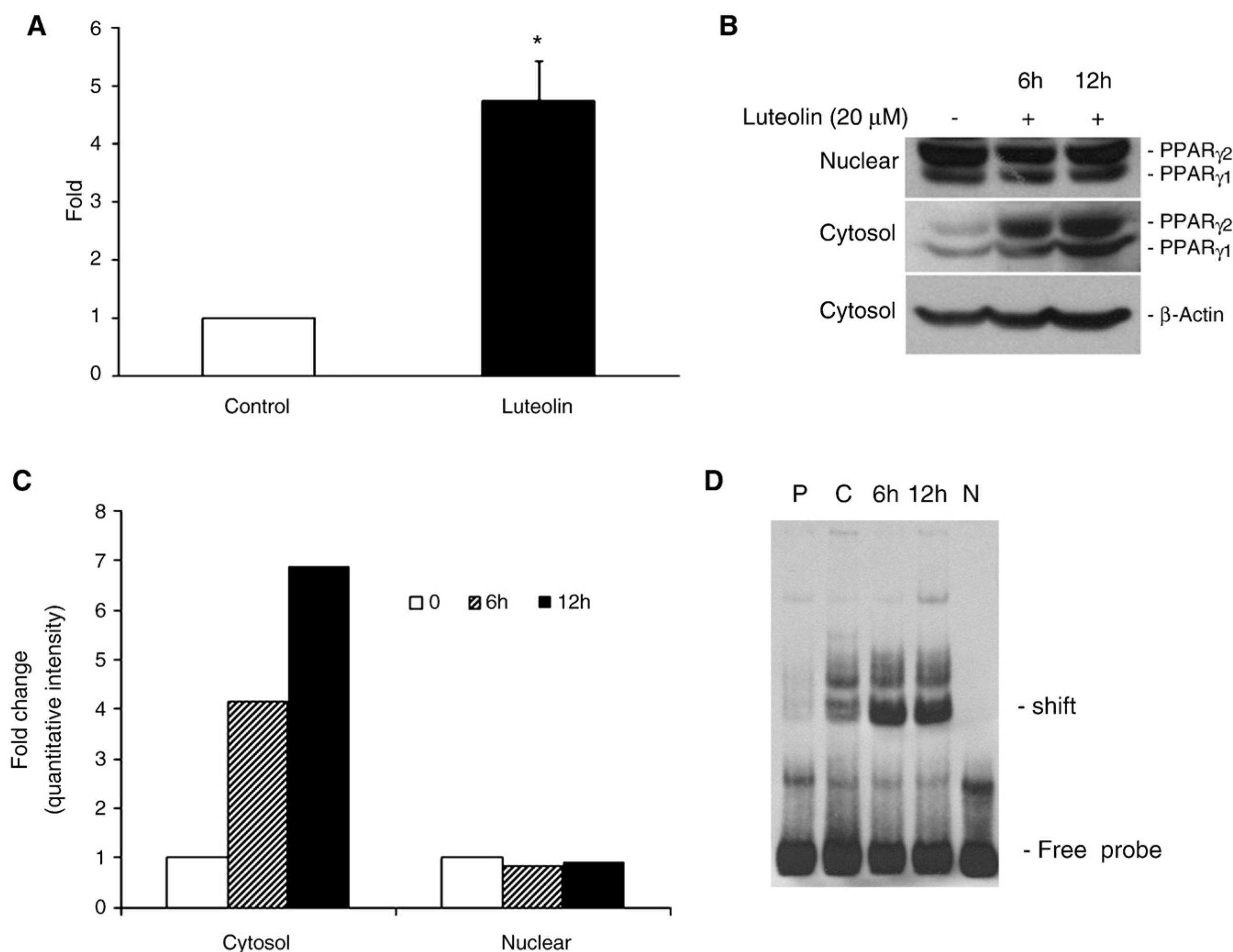

Fig. 4. Luteolin induces PPARγ expression and PPARγ DNA binding activity in 3T3-L1 adipocytes. Day-8-differentiated 3T3-L1 adipocytes were treated with 0.5% BCS and 1 mg/ml glucose for 12 h. Cells were treated with or without luteolin (20 μmol/L) for 24 h. Total RNAs were extracted to determine the mRNA levels of PPARγ (A). For examination of PPARγ nuclear activation, cells were treated with or without luteolin (20 μmol/L) for 6 and 12 h, respectively. Nuclear and cytosolic proteins were extracted for Western blot analysis (B). The signals of PPAR proteins were quantified and presented as fold change of luteolin-treated versus control cells (C). The DNA binding activity of nuclear PPARγ was determined by EMSA (D). Lane N: negative control containing only labeled DNA probe with no nuclear proteins. Lane P contains a mix of nuclear proteins and labeled DNA probe, plus 200-fold unlabeled DNA probe. Lane C contains nuclear proteins from control 3T3-L1 adipocytes and labeled DNA probe. Lanes 6 h and 12 h contain labeled DNA probe and nuclear proteins extracted from 3T3-L1 adipocytes treated with luteolin for 6 and 12 h, respectively. The results in (A) are the mean±S.E. of three independent experiments. The results in (B) and (C) represent triplicates of cultures (*P<.05, luteolin treatment vs. control).

treated with or without luteolin for 6 and 12 h, respectively. As illustrated in Fig. 4B and C, protein levels of nuclear PPARγ were not significantly changed regardless of luteolin treatment. However, luteolin treatment for 6 and 12 h caused a marked increase in the levels of cytosol PPARγ protein. To test the possible effect of luteolin on PPARγ transcriptional activation, we determined the binding activity of PPARγ to PPRE in differentiated 3T3-L1 adipocytes treated with or without 20 μmol/L luteolin for 6 and 12 h. The result of EMSA showed that both 6-h and 12-h treatment of luteolin dramatically enhanced the binding of PPARγ to PPRE consensus (Fig. 4D), indicating an increased PPARγ transcriptional activity.

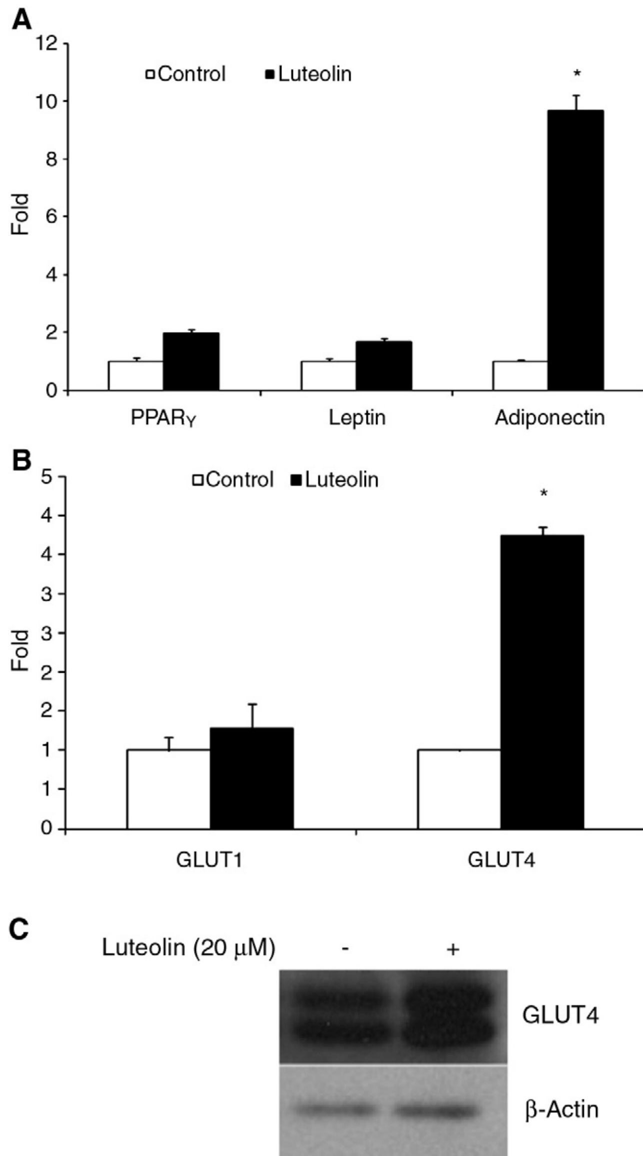

Fig. 5. Effects of luteolin on the gene expression of PPARγ, adipokines and GLUTs in primary adipose cells. Primary adipose cells were isolated from the epididymal adipose tissue of four normal C57BL/6J mice by collagenase digestion, as described in Materials and Methods. Cells were then cultured in 3.5% BSA DMEM in the presence or in the absence of 20 μmol/L for 6 h. Total RNAs were extracted to determine the mRNA levels of PPARγ, leptin and adiponectin (A), as well as GLUT1 and GLUT4 (B). Primary adipose cells isolated from the epididymal adipose tissue of six C57BL/6J mice were cultured in the absence or in the presence of 20 μmol/L luteolin for 6 h. Total cell lysates were harvested for GLUT4 protein expression (C). The results represent the mean±S.E. of triplicates of cultures of adipose cells isolated from four C57BL/6J mice (*P<.05, luteolin treatment vs. control).

To further investigate whether PPARγ activation is important for mediating luteolin effects on adipocytes, we added GW9662 (a PPARγ antagonist), together with luteolin, to the culture medium of 3T3-L1 adipocytes, and the gene expression of adiponectin and leptin was examined. As shown in Fig. 3A, luteolin treatment alone for 24 h significantly increased the gene expression of adiponectin and leptin. However, the addition of GW9662 significantly blocked the luteolin-induced gene expression of adiponectin and leptin.

3.4. Effect of luteolin on the gene expression of PPARγ, adipokines and GLUTs in primary mouse adipose cells

To verify the truly biological relevance of the above observations in 3T3-L1 adipocytes, we used primary mouse adipose cells that reflect biological responses better to examine the effects of luteolin on the gene expression of PPARγ, adiponectin and leptin. Primary adipose cells were isolated from normal C57BL/6J mice and cultured in DMEM in the presence or in the absence of luteolin for 6 h. Fig. 5A showed that luteolin significantly enhanced the gene expression of adiponectin, leptin and PPARγ_2 (P<.05) in primary adipose cells, similar to that in 3T3-L1 adipocytes.

GLUT4 and GLUT1 are the two predominant GLUTs in adipose cells. GLUT1 is localized on the plasma membrane of cells and is mainly responsible for basal glucose transport, while GLUT4 primarily contributes to insulin-stimulated glucose transport. To address whether changes in the levels of GLUT1 and GLUT4 gene expression could contribute to the luteolin effect on glucose uptake, we examined the gene expression of GLUT 1 and GLUT4 in primary mouse adipose cells. As demonstrated in Fig. 5B, the levels of GLUT1 gene expression were not significantly different between control cells and cells treated with luteolin for 6 h, whereas the levels of *GLUT4* gene (Fig. 5B) and protein (Fig. 5C) expression were significantly increased in luteolin-treated adipose cells.

4. Discussion

Flavonoids are a group of phenylbenzopyrones that possess anti-oxidant, anti-carcinogenic and anti-inflammatory activities. As a member of the flavonoid family, luteolin has been shown in a number of previous studies to protect cells from LPS-induced cytokine release and inflammatory responses. Herein, we demonstrate the effect of luteolin on insulin action in adipocytes.

First, we examined the effect of luteolin on glucose uptake in adipocytes. The results showed that luteolin significantly increased the response of glucose uptake to insulin stimulation in 3T3-L1 adipocytes. The mechanism for the effect of luteolin on glucose uptake could be speculated by directly interacting with GLUTs on the cell membrane or by indirectly affecting insulin action and the levels of gene expression of GLUTs. Several studies have reported that flavonoids act as competitive inhibitors of glucose uptake in other types of cells [23]. A group of flavonoids, such as genistein, quercetin, myricetin, morin, rhamnetin and isohamnetin, was observed to have inhibitory effects on GLUT1-mediated glucose uptake in erythrocytes, U937 cells and HL-60 cells [24–26]. This inhibition was claimed to be the result of a direct interaction of flavonoids with the membrane GLUT1 transporter based on the observation that flavonoids competed with glucose for the binding of GLUT1 [24]. Our result showed that luteolin caused a slight decrease (no statistical significance) in the basal levels of glucose uptake in 3T3-L1 adipocytes, suggesting that luteolin may interfere with GLUT1-mediated glucose transport in adipocytes, as GLUT1 is the major GLUT on the cell surface of adipocytes in the basal state. However, this suggestion was not supported by the result that GLUT1 gene expression was not significantly affected by luteolin in primary mouse adipose cells. In the insulin-stimulated state, GLUT4 is translocated to the cell surface

and becomes the predominant GLUT in response to insulin stimulation. In addition to increased insulin-stimulated glucose uptake and Akt Ser473 phosphorylation, the treatment with luteolin led to a marked increase in GLUT4 gene expression in primary mouse adipose cells. Thus, our data support that luteolin affects glucose uptake via mechanisms involving increased insulin signaling activity and GLUT4 expression level.

In contrast to our finding, Park [24] showed that other members of the flavonoid family, such as myricetin, quercetin and catechin gallate, inhibit GLUT4-mediated glucose uptake in isolated rat adipocytes. Possible explanations for this discrepancy could be as follows: First, the potential bioactivities of flavonoids are mostly dependent on their uptake and metabolism in the cells. Flavonoids exert their bioactive functions via distinctive metabolites in different cell types [27]. Therefore, the effect of the flavonoids myricetin, quercetin and catechin gallate on GLUT4-mediated glucose uptake in their study cannot be simply compared to the effect of luteolin tested in our study. Second, the experimental designs are very different between the two studies in terms of cell models, concentrations and treatment time periods of flavonoids. In their study, primary rat adipocytes were used to examine the acute (30 min) effect of much higher concentrations of flavonoids. Thus, the distinct effects of luteolin on glucose uptake observed in the two studies could possibly be related to the abovedescribed reasons.

Most of the previous studies focused on the anti-inflammatory properties of flavonoids in macrophages. The anti-oxidant and anti-inflammatory properties of flavonoids have been attributed partially to their chemical structure characteristics (e.g., as a hydrogen donor/reducing agent). The structure variations of flavonoids may explain the difference in their anti-inflammatory bioactivities. Luteolin has been shown to have a double bond at positions C2–C3 of the C ring and OH groups and at positions 3′ and 4′ of the B ring that confer to luteolin the most potent anti-inflammatory activity [13,28]. However, recent studies have speculated that the structure characteristic of flavonoids is unlikely to be the sole explanation for their cellular effects. In particular, the interactions of flavonoids with specific proteins central to intracellular signaling cascade have become increasingly important.

A previous study on the intestinal absorption of luteolin in rats showed that luteolin could be converted into glucuronides while passing through the intestinal mucosa [27]. Four hydroxyl groups of luteolin are available for glucuronidation, and the presence of hydroxyl groups is considered to be directly associated with the anti-inflammatory activity of luteolin. However, the main metabolite of luteolin in plasma is a monoglucuronide, although some luteolin can escape intestinal conjugation and hepatic sulfation/methylation. Theoretically, a monoglucuronide with only one hydroxyl group might lower its structure-related anti-inflammatory activities. Therefore, the cellular effect of luteolin is more likely related to its action at intracellular signaling cascades. Flavonoids and their metabolites have been reported to affect a number of protein and lipid kinase signaling cascades such as Akt/protein kinase B, tyrosine kinases, protein kinase C and mitogen-activated protein kinase (MAPK) pathways [27]. An *in vitro* study showed that luteolin reduced LPS-induced TNFα release by inactivating extracellular-regulated kinases, p38 MAPK and casein kinase 2 in macrophages [28]. In another study, luteolin inhibited TNFα-induced IL-8 production by attenuating MAPK phosphorylation, IκBα degradation and NF-κB activation in human intestinal epithelial cells [29].

As an endocrine organ, adipose tissue secretes a variety of adipokines and cytokines that exert regulatory functions in insulin resistance and energy metabolism. This secretory function is altered in obesity, contributing to impaired adipocyte metabolism and an inflammatory state that is associated with insulin resistance. In this study, we also investigated the possible effect of luteolin on adipocyte secretory activity. Luteolin treatment markedly enhanced the mRNA levels of gene expression for two key adipokines, leptin and adiponectin, whereas it significantly reduced the gene expression of the proinflammatory cytokines TNFα, IL-6 and MCP-1. PPARγ is a well-known key transcription factor controlling adipogenesis, metabolism and insulin sensitivity. PPARγ is also a key therapeutic target for type 2 diabetes. The compounds that could affect PPARγ activity possess a huge potential for developing preventive and therapeutic agents for diabetes. Glucose uptake is one of the important readouts of insulin sensitivity in adipocytes. However, adipose tissue accounts for only 15–20% of insulin-dependent glucose disposal, as compared to ~80% in skeletal muscle. It may or may not be directly regulated by PPARγ activity in adipocytes. In contrast to glucose uptake, PPARγ activity and its controlled adipokine production are more important contributors to the regulation of systemic insulin sensitivity. PPARγ activity is a more important phenomenon in the evaluation of the insulin-sensitizing effects of compounds.

Adiponectin and *leptin* are two important PPARγ target genes. Activation of PPARγ by TZD induces adiponectin gene expression in 3T3-L1 adipocytes [22], while PPARγ activation has an inverse correlation with leptin gene expression, as reported in previous studies [30–33]. GW9662 is known as a potent and selective PPARγ antagonist and has been used to specifically block PPARγ activation in many studies [34–36]. Although it has also been reported that GW9662 might act through other PPARγ-independent pathways, selectively antagonizing PPARγ activity is the primary action of GW9662 [37]. In this study, GW9662 was used to test whether PPARγ activation is important for luteolin effect on adiponectin and leptin expression. Consistently, we observed that luteolin induces the expression of PPARγ and adiponectin; the addition of GW9662, a PPARγ antagonist, blocked luteolin-induced adiponectin expression, suggesting that PPARγ is a possible direct target of luteolin in adipocytes. PPARγ, as a target of other natural compounds such as phytol and abietic acid, has also been reported in other studies [38,39]. Additionally, we examined the effect of luteolin on PPARγ nuclear translocation and PPARγ DNA binding activity to further elucidate the possible mechanism for its effect on insulin action. Our data demonstrated that the distribution of PPARγ protein in nuclear fraction was unchanged, while cytosolic PPARγ was dramatically increased in response to luteolin treatment. The similar observation has been reported in a previous investigation of the effect of pioglitazone (PIOG) on adipose PPARγ_2 protein [40]. In that study, the distribution of PPARγ_2 in nuclear and cytosolic fractions was not altered by treatment with PIOG, a PPARγ ligand, in 3T3-L1 adipocytes [40]. However, the binding of PPARγ_2 to the DNA-binding element of A-FABP was significantly enhanced by 12-h treatment with PIOG [40], suggesting that nuclear translocation is not an important mechanism for PPARγ activation. Similarly, our EMSA data showed a significant increase in the binding of PPARγ to PPRE in luteolin-treated 3T3-L1 adipocytes. Therefore, increased cytosolic PPARγ and nuclear PPAR DNA binding activity suggest that luteolin may increase PPARγ activity through two mechanisms by increasing the total expression levels of PPARγ and by enhancing PPARγ transcriptional activation.

All the above data support that increased PPARγ transcriptional activity is the primary effect of luteolin. However, the results of GW9662 inhibition on luteolin-induced leptin expression seem not to support the PPARγ activation model because PPARγ activation is associated with decreased leptin gene expression, as reported in many studies [30–33]. Thus, the other PPARγ-independent mechanisms cannot be ruled out. Indeed, the concept for multiple targets of luteolin effects has been suggested by a previous study, which states that citrus auraptene and isoprenols from herbal medicine have been shown to serve as dual agonists of both PPARα and PPARγ [41,42]. Therefore, PPARγ activation, together with increased GLUT4

expression and activation of insulin signaling cascade, could contribute to the insulin-sensitizing effect of luteolin in adipocytes.

In conclusion, our data demonstrate that luteolin potentiates insulin action and increases expression and transcriptional activation of PPARγ and expression of the PPARγ target genes *adiponectin*, *leptin* and *GLUT4* in 3T3-L1 adipocytes, as well as in primary mouse adipose cells, and that PPARγ antagonist inhibits this induction. Our findings suggest that luteolin potentiates insulin action in adipocytes by directly activating the PPARγ pathway and by acting at insulin signaling cascade. However, further *in vivo* study of the anti-diabetic effect of luteolin is necessary to elucidate its pharmaceutical potential and ability.

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