Pollen Typhae total flavone improves insulin-induced glucose uptake through the β-arrestin-2-mediated signaling in C2C12 myotubes

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Abstract. Defects in insulin-stimulated glucose uptake in skeletal muscle result from the dysfunction of insulin signaling including the phosphatidylinositol-3 kinase (PI3K) pathway and a novel β-arrestin-2-mediated signaling, which leads to insulin resistance (IR). Pollen Typhae, a Chinese herb, has been used for thousands of years in traditional Chinese medicine, and has the potential to inhibit the development of IR. We have previously reported that Pollen Typhae total flavone (PTF), the extract from Pollen Typhae, ameliorates high-glucose- and high-insulin-induced impairment of glucose uptake in 3T3-L1 adipocytes, but the mechanisms remain unclear. The objective of this study was to investigate the effects of PTF on glucose uptake, and to explore the underlying mechanisms in C2C12 myotubes. PTF improved insulin-stimulated glucose uptake in a dose- and time-dependent manner in C2C12 myotubes, and prevented palmitate-induced IR. Furthermore, PTF enhanced the basal gene expression of Src and Akt2, elevated the protein expression of β-arrestin-2, Src and Akt, increased the phosphorylation of insulin receptor-β at Tyr1150/1151 and Akt at Thr308/Ser473 in an insulin-dependent manner, but had no effects on the protein expression of PI3K-p85 or the activity of PI3K. Inhibition of Src but not PI3K restrained PTF-induced phosphorylation of Akt and glucose uptake. Our findings indicate that PTF improves insulin-induced glucose uptake via the β-arrestin-2-mediated signaling in C2C12 myotubes.

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Introduction

As the largest insulin-sensitive tissue, skeletal muscle is considered the most important tissue for insulin-mediated glucose disposal, accounting for approximately 80% of insulin-stimulated glucose uptake (1). Defects in insulin-induced glucose uptake in the tissue are strongly linked to insulin resistance (IR) (2), a hallmark of metabolic diseases including type 2 diabetes and the metabolic syndrome. The prevalence of these conditions continues to rise thus imposing an enormous healthcare burden worldwide (3,4).

IR is simply the inability of insulin to stimulate insulin signaling. Under insulin stimulation, the activated insulin receptor (InsR) recruits and phosphorylates insulin receptor substrate proteins (IRS), causing activation of the phosphatidylinositol-3 kinase (PI3K) that phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-triphosphate (PIP_3) which recruits phosphoinositide-dependent kinase 1/2 (PDK1/2) and the three known isoforms of Akt/protein kinase B (PKB), and then PDK1 and PDK2 phosphorylate Akt/PKB on Thr308/Ser473 respectively. Activated Akt/PKB regulates downstream targets such as glucose transporter-4 (Glut4), glycogen synthase kinase 3 (GSK3), forkhead box O1 (Foxo1), hormone sensitive lipase (HSL), and mTOR, thus being responsible for most of the metabolic actions of insulin to maintain glucose, fat and protein homeostasis, including glucose uptake, glucose synthesis, and gluconeogenesis (5-9). It is well known that the inactivation of the PI3K pathway leads to IR, showing a decrease in insulin-induced glucose uptake and disposal (10,11). Recently, a novel insulin signaling dependent on β-arrestin-2 has been discovered (12). Upon stimulation by insulin, β-arrestin-2 scaffolds Src and Akt to InsR, causing the

formation of a new β-arrestin-2 signal complex, which allows Src to phosphorylate Akt, thus enhancing the phosphorylation of Akt at Thr308/Ser473, and subsequently regulating insulin-mediated phosphorylation of GSK3 and Foxo1, promoting insulin-stimulated translocation of Glut4 from intracellular organelles (endosomes) to the cell surface within insulin-responsive tissues, where Glut4 binds glucose and is in charge of glucose uptake (12,13). Loss or dysfunction of β-arrestin-2 leads to deficiency of this complex and disturbance of the signaling, resulting in IR and progression of type 2 diabetes. On the contrary, overexpression of β-arrestin-2 promotes the formation of the complex, and improves insulin sensitivity in insulin resistance model animals (12,14-16). β-arrestin-2 and the β-arrestin-2-mediated signaling display a potential role in preventing IR. Therefore, a strategy for enhancing glucose uptake is to preserve or strengthen the β-arrestin-2-mediated signaling.

Pollen Typhae is the pollen of several species of the genus *Typha* (Typhaceae) including *T. angustifolia* L., *T. orientalis* Presl., *T. davidiana* Hand.-Mazz. and *T. minima* Funk. It has been widely used to treat trauma, haematemesis, metrorrhagia, dysmenorrhea, hematuria and stranguria in Chinese medical clinical practice. Increasing evidence also indicates that Pollen Typhae performs a series of pharmacological functions. For example, Pollen Typhae improves the microcirculation, ameliorates dyslipidemia, and prevents and controls coronary heart diseases and myocardial infarction (17,18). Moreover, it shows cytotoxicity against tumor cells (19), and regulates immune activity (20). Studies have proven that the main constituents of Pollen Typhae contain flavones, linoleic acid, and other unsaturated fatty acids (21,22). Pollen Typhae total flavone (PTF), the extract of Pollen Typhae, possesses anti-inflammatory and anti-coagulant activities (23,24). In clinical research and animal studies, the Chinese herbal medicine 'Yiqi Sanju Formula' chiefly consisting of Pollen Typhae and several other Chinese herbs has been used to treat type 2 diabetes, central obesity, and non-alcoholic fatty liver disease, characterized by IR, showing anti-IR activity (25-27). We have also reported that PTF ameliorated high-glucose- and high-insulin-induced impairment of glucose uptake in 3T3-L1 adipocytes (28), suggesting the ability of PTF to improve IR, but the potential molecular mechanisms remain unclear.

The aim of this study was to investigate the effects of PTF on glucose uptake, and to explore the underlying molecular mechanisms in C2C12 myotubes.

Materials and methods

Reagents. Dulbecco's modified Eagle's medium (DMEM), fetal calf serum (FCS), and horse serum were purchased from Gibco (Grand Island, NY). PP2, wortmannin, insulin solution, bovine serum albumin (BSA), palmitic acid, 2,3-bis(2-methoxy-4-nitro-5-sulfophenyl)-5-[(phenylamino) carbonyl]-2H-tetrazolium hydroxide (XTT), and cytochalasin B were obtained from Sigma (St. Louis, MO), fatty acid-free BSA was from Roche (Mannheim, Germany). Culture plates were from Corning (New York, NY). 2-Deoxy-D-[2,6-^{3}H] glucose (2-DOG) was obtained from Amersham (Buckinghamshire, UK). Antibodies directed against Akt (total), Akt (phosphorylated Thr308), Akt (phosphorylated Ser473), and PI3K-p85 were from Cell Signaling Technology (Danvers, MA). Secondary HRP-conjugated antibodies and antibodies to InsR-β (phosphorylated Tyr1150/1151), Src, and β-arrestin-2 were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). The PI3K ELISA kit was from Echelon Biosciences and BCA protein assay kit was from Pierce (Rockford, IL). TRIzol reagents were purchased from Invitrogen Life Technologies (Carlsbad, CA).

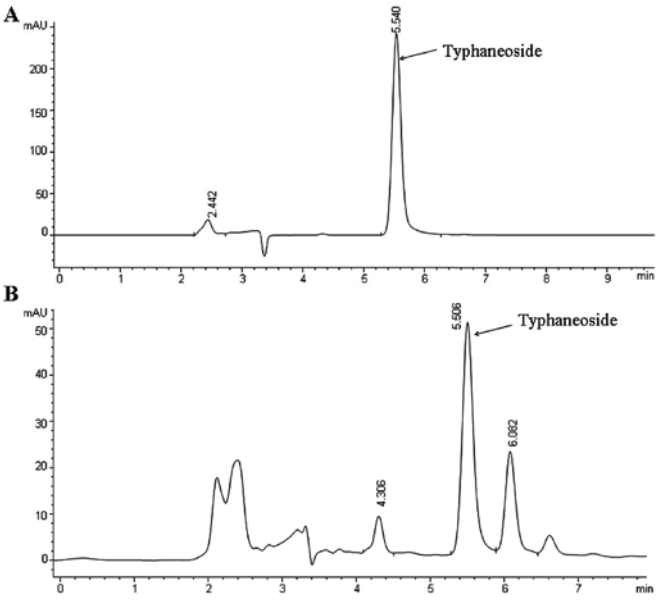

Figure 1. Chemical profiles of PTF by HPLC. (A) Typhaneoside. (B) Sample.

Preparation of PTF. The pollen of *T. angustifolia* L. was collected in Anguo, Hebei, China, in June 2004, and authenticated by W.-J.W. based on microscopic and macroscopic characteristics. The fresh pollen of *T. angustifolia* L. was dried at 37˚C with protection from light, and ground into powder. The extract of pollen of *T. angustifolia* L. was obtained as described with some modifications (20,21). Briefly, the material (500 g) was extracted with 70% ethanol three times under reflux for 1 h. After filtration and concentration in rotavapour at 45˚C until there was no flavor of ethanol, the solution was extracted with n-butyl alcohol three times. The combined solution was then concentrated under reduced pressure in rotavapor at 45˚C to evaporate the solvent, and finally dried in high vacuum. The dried extract weighed 8.78 g (yield 1.76%, w/w), which was determined to be PTF by HPLC according to the study by Yang *et al* (21), which contains Typhaneoside and undefined components (Fig. 1). The specimens of dried pollen of *T. angustifolia* L. and extract were deposited in the Institute of Chinese Integrative Medicine, Huashan Hospital, Fudan University, Shanghai, China.

Cell culture and differentiation. C2C12 myoblasts were obtained from the American Type Culture Collection (Manassas, VA). The cells were maintained in DMEM supplemented with 10% (vol/vol) FCS and antibiotics (penicillin 100 U/ml, streptomycin 100 μg/ml) for growth at 37˚C under a humidified atmosphere containing 5% CO_2. For differentiation of myotubes, C2C12 myoblasts were transferred to DMEM containing 2% horse serum when the cells reached confluence.

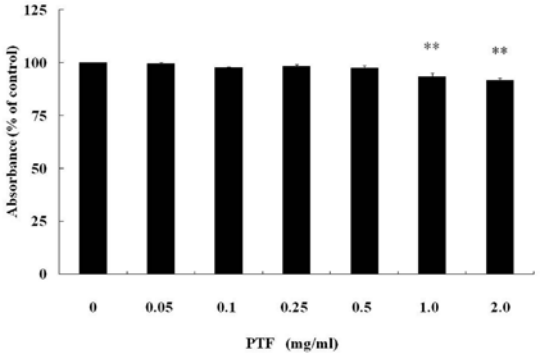

Figure 2. Cytotoxicity of PTF in C2C12 myotubes. C2C12 myotubes in 96-well culture plates were treated with PTF for 24 h after serum starvation in 1% BSA-DMEM. Cytotoxicity was detected by the XTT assay. n=7, **P<0.01 vs. control (0 mg/ml).

After an additional 4 days, the cells fused into myotubes, which were used in the subsequent experiments. The medium was changed every other day.

Cytotoxicity analysis. The cytotoxicity of PTF was determined by the XTT assay (29). C2C12 myoblasts were cultured in 96-well culture plates. After differentiation, the medium was replaced by serum-free DMEM supplemented with 1% BSA, and the cells were cultured for 12 h. C2C12 myotubes in each well were then treated with 100 μl serum-free 1% BSA-DMEM containing different concentrations of PTF (0, 0.05, 0.1, 0.25, 0.5, 1.0 and 2.0 mg/ml). After treatment for 24 h, 50 μl of 0.1% XTT dissolved in serum-free DMEM was added directly into each well. After incubation for 4 h at 37˚C, the optical absorbance was read on a microplate reader (ELX 800; Bio-Tek Instruments, Winooski, VT) at 490 nm.

Glucose uptake assay. Glucose uptake was determined by measuring the transport of 2-DOG into the cells as previously described with some modifications (30). C2C12 myoblasts were differentiated in 24-well culture plates. After 12-h serum deprivation and then PTF treatment for different times (8, 16 and 24 h), the cells were washed three times with Krebs-Ringer phosphate buffer (KRPB) (10 mM HEPES, 131.2 mM NaCl, 4.7 mM KCl, 1.2 mM $MgSO_4$, 2.5 mM $CaCl_2$, 2.5 mM NaH_2PO_4, pH 7.4), and then incubated in KRPB with or without 100 nM insulin at 37˚C. After 20 min, 0.5 μCi/ml 2-DOG was added to the cells. After 10 min incubation, the cells were washed three times with ice-cold PBS containing 10 mM glucose to terminate the reaction. Finally, the cells were lysed with 0.1 N NaOH for 2 h, and the radioactivity taken up by the cells was measured by a scintillation counter (Beckman Instruments). Non-specific glucose uptake was determined in the presence of 20 μM cytochalasin B, and this was subtracted from the total uptake to get specific glucose uptake.

Western blotting and ELISA assay. After 12-h serum deprivation, C2C12 myotubes in 6-well plates were treated with PTF for 16 h, and then incubated with or without 100 nM insulin for 30 min. The cells were washed three times with ice-cold PBS, and then lysed in cell lysis buffer (RIPA, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 1% NP-40, 1 mM PMSF, 2 μg/ml pepstatin, 10 μg/ml leupeptin, pH 7.4). The insoluble lysate material was removed by centrifugation (12,000 rpm for 30 min, 4˚C), and the protein concentrations of supernatants were determined using a BCA protein assay kit, which were utilized for western blotting and ELISA as previously described (31). The cell lysates were boiled for 5 min, and then separated via SDS-PAGE. The protein was electrophoretically transferred to polyvinylidene difluoride membranes. The membranes were incubated with the first antibody for 1-2 h and then the secondary antibody for 1-2 h. Immunoreactive bands were visualized by incubation with ECL Plus detection reagents (Amersham Biosciences). For the measurement of PI3K activity, lysates were measured by ELISA per the manufacturer's instructions. The detection limits were 12.5 to 200 pmol in 100 μl detection volume.

Quantification of mRNA levels by real-time PCR. The mRNA levels of β-arrestin-2, Src, and Akt2 were identified by real-time PCR after reverse transcription as previously described (32). After 12-h serum deprivation, C2C12 myotubes in 6-well culture plates were treated with PTF for 16 h, and then washed three times with ice-cold PBS. Total-RNA was extracted from the cells using TRIzol reagents, and then transcribed into cDNA with a superscript first-strand cDNA synthesis system. The relative gene abundance was quantified by real-time PCR, the reactions were performed in an ABI 7500 sequence detection system. The sequences of primers used were: β-arrestin-2, forward, 5'-TCC CTA GGG CGG CAA GCT GT-3' and reverse, 5'-ACT GGG GGC GAG TTG GTG TGA-3'; Src, forward, 5'-TCG GAC ACC GTC ACC TCC CC-3' and reverse, 5'-GAC AAT CTG CAG CCG CTC CCC-3'; Akt2, forward, 5'-AAA AAG TGG CTC TGG TGT GTG GAG C-3' and reverse, 5'-GAC TGT GGT CCA CTG CAG GCA-3'; GAPDH, forward, 5'-CCC CAG CAA GGA CAC TGA GCA AGA G-3' and reverse, 5'-GCC CCT CCT GTT ATT ATG GGG GTC-3'.

Statistical analysis. The data are presented as means ± standard error (SE). Changes in 2-DOG uptake between baseline and insulin stimulation (known as 2-DOG uptake induced by insulin) are expressed as δ. All data were analyzed with SPSS 16.0 for Windows. To identify significant differences between the two groups, comparisons were analyzed by the Student's t-test. When multiple comparisons were performed, the significance was analyzed by one-way analysis of variance (ANOVA). A value of P<0.05 was regarded as statistically significant.

Results

Cytotoxicity of PTF in C2C12 myotubes. The cytotoxicity of PTF in C2C12 myotubes was examined after PTF treatment for 24 h (Fig. 2). In C2C12 myotubes, PTF treatment decreased cell activity in a dose-dependent manner. PTF doses of 0.05-0.5 mg/ml, were not cytotoxic, but PTF was cytotoxic (P<0.01) at the concentration of 1.0 mg/ml or above compared with the control (0 mg/ml).

PTF ameliorates insulin-stimulated glucose uptake in a dose- and time-dependent manner in C2C12 myotubes. Glucose uptake was investigated for PTF in C2C12 myotubes with 2-DOG. In the absence of insulin, 2-DOG uptake was not promoted with PTF treatment for 8, 16 or 24 h (Fig. 3A, C

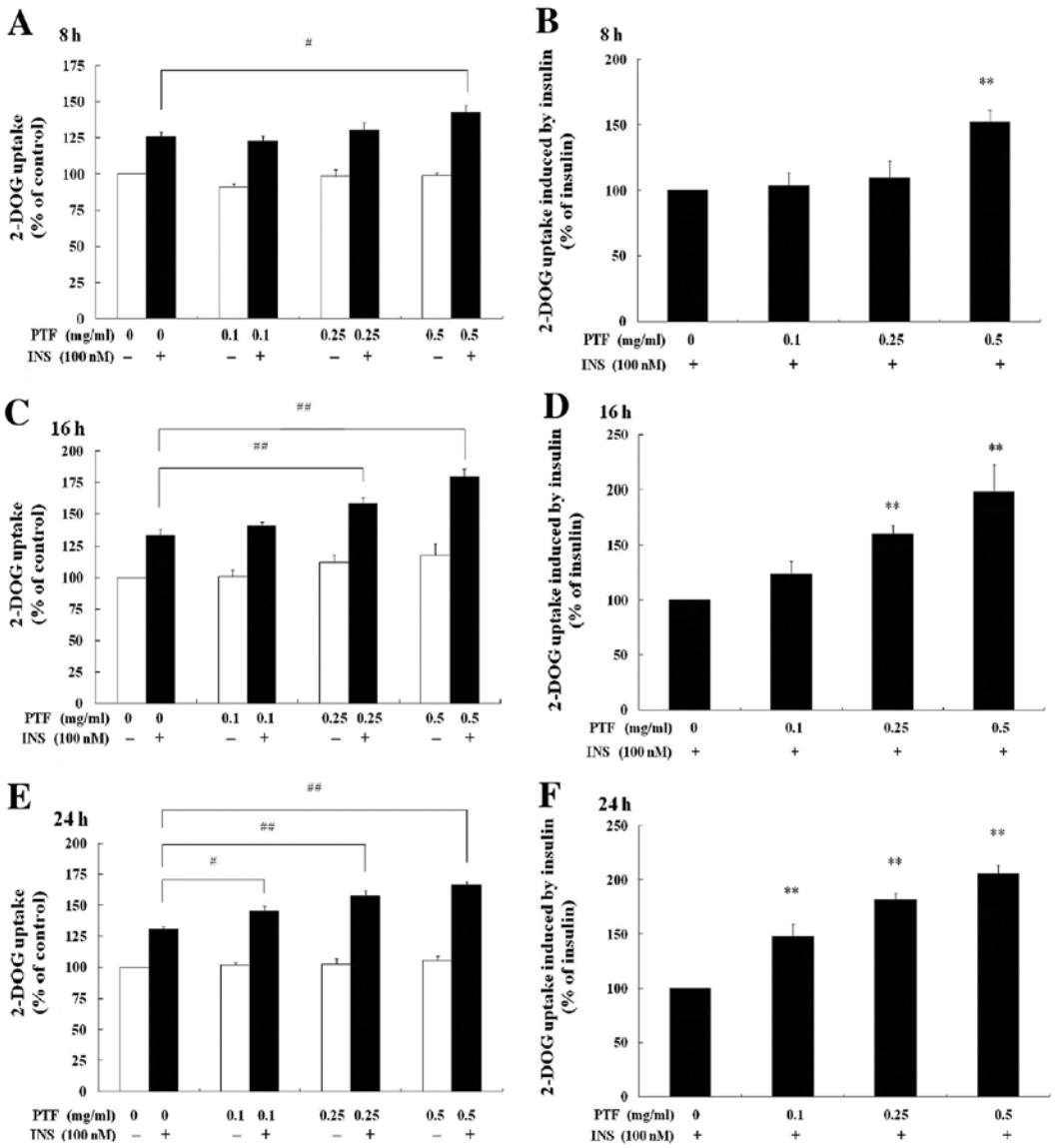

Figure 3. Effects of PTF on glucose uptake in C2C12 myotubes. After serum starvation in 1% BSA-DMEM and PTF pre-treatment for the indicated times, C2C12 myotubes in 24-well culture plates were treated with or without insulin (INS, 100 nM) for 20 min and glucose uptake was determined with 2-DOG. (A) 2-DOG uptake after 8 h. (B) 2-DOG uptake induced by insulin after 8 h. (C) 2-DOG uptake after 16 h. (D) 2-DOG uptake induced by insulin after 16 h. (E) 2-DOG uptake after 24 h. (F) 2-DOG uptake induced by insulin after 24 h. n=6, $^{\#}$P<0.05, $^{\#\#}$P<0.01; **P<0.01 vs. INS alone.

and E) at all concentrations (0.1, 0.25 or 0.5 mg/ml) compared with the control (0 mg/ml PTF). In the presence of insulin, compared with insulin alone, only 0.5 mg/ml PTF increased insulin-induced 2-DOG uptake by 52.26% (P<0.01) after PTF pre-treatment for 8 h (Fig. 3B). After PTF pre-treatment for 16 h (Fig. 3D), both 0.25 and 0.5 mg/ml PTF dramatically improved insulin-induced 2-DOG uptake by 60.00% (P<0.01) and 98.76% (P<0.01) respectively. Additionally, after PTF pre-treatment for 24 h (Fig. 3F), PTF at all concentrations of 0.1, 0.25 and 0.5 mg/ml prominently enhanced insulin-induced 2-DOG uptake by 47.60% (P<0.01), 81.46% (P<0.01) and 105.82% (P<0.01), respectively. These results indicate that PTF improves glucose uptake in an insulin-dependent manner in C2C12 myotubes, suggesting the potential ability of PTF to ameliorate IR.

PTF prevents palmitate-induced IR in C2C12 myotubes. According to Reaven's initial studies (33), IR is defined as a decrease in insulin-mediated glucose uptake. In our previous study (28), PTF was established to improve high-glucose- and high insulin-induced IR in 3T3-L1 adipocytes. In order to determine whether PTF ameliorate IR in C2C12 myotubes, palmitate (PA) was used to induce IR according to the study by Senn (31). PA (0.5 mM) pre-treatment for 16 h resulted in a reduction in insulin-induced glucose uptake by 63.69% (P<0.01) in C2C12 myotubes compared with insulin alone (Fig. 4B). After both PTF and PA pre-treatment for 16 h, PTF (0.5 mg/ml) notably increased insulin-mediated glucose uptake (P<0.01) compared with PA + insulin (Fig. 4D), which was similar to insulin alone (P>0.05).

PTF has no effects on the protein expression of p85 or the activity of PI3K in C2C12 myotubes. To gain insight into the molecular mechanisms, we observed the effects of PTF on insulin signaling. PI3K, which consists of a regulatory (p85) and a catalytic subunit (p110), and plays a key role in the PI3K

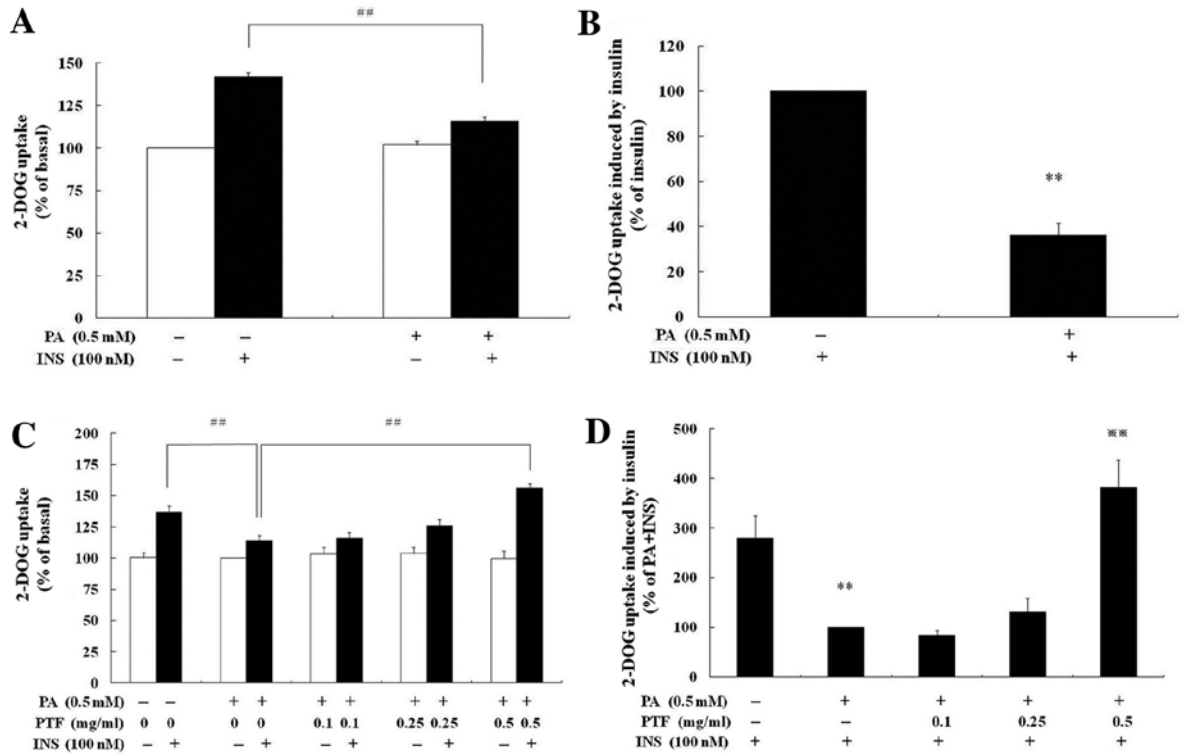

Figure 4. Effects of PTF on palmitate-induced insulin resistance in C2C12 myotubes. After serum starvation in 1% BSA-DMEM, C2C12 myotubes in 24-well culture plates were treated with palmitate (PA) and/or PTF for 16 h, and then incubated with or without insulin for 20 min. Glucose uptake was determined with 2-DOG. (A) Effects of PA on 2-DOG uptake. (B) Effects of PA on insulin-induced 2-DOG uptake. (C) Effect of PTF on PA-induced 2-DOG uptake. (D) Effect of PTF on insulin + PA-induced 2-DOG uptake. n=6, $^{\#\#}$P<0.01; **P<0.01 vs. INS alone; **P<0.01 vs. PA + INS.

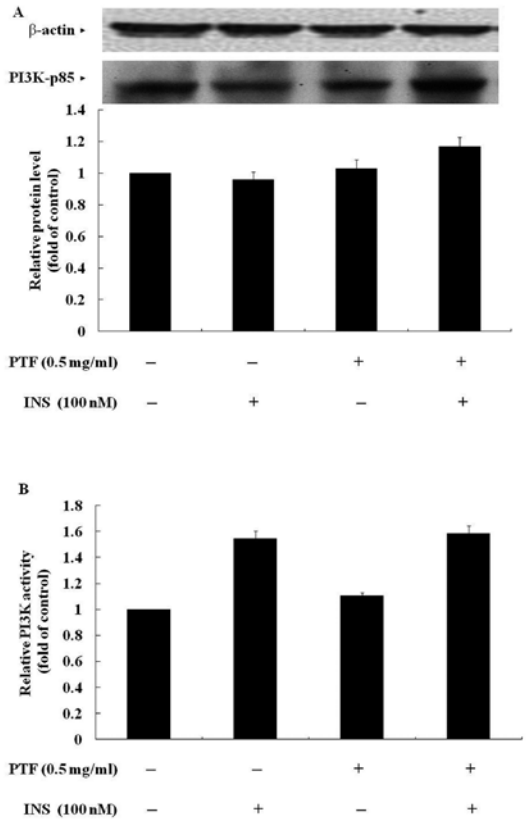

Figure 5. Effects of PTF on the protein expression of p85 and the activity of PI3K in C2C12 myotubes. Serum-starved C2C12 myotubes were treated with or without PTF for 16 h and then with or without insulin for 30 min. Cells were lysed, and (A) the protein expression of p85 and (B) the activity of PI3K were determined by western blotting and ELISA, respectively.

pathway, was firstly investigated. Western blotting showed that C2C12 myotubes were pre-treated with PTF (0.5 mg/ml) for 16 h, the protein expression of p85 was not obviously changed whether there was insulin to stimulate the cells for 30 min or not (Fig. 5A). The ELISA assay revealed that the activity of PI3K was not induced with PTF treatment for 16 h in the absence of insulin. In the presence of insulin, insulin obviously increased the activity of PI3K, and PTF pre-treatment for 16 h did not further enhance the activity of PI3K (Fig. 5B). These data indicate that PTF exhibits few effects on the protein expression of p85 and the activity of PI3K, suggesting that PTF improves insulin-induced glucose uptake independent of PI3K.

PTF improves the phosphorylation of InsR-β in an insulin-dependent manner in C2C12 myotubes. Tyrosine autophosphorylation of InsR, comprised of two extracellular α subunits and two intracellular β subunits, is one of the earliest cellular responses to insulin stimulation. Autophosphorylation begins with the phosphorylation of β subunits at Tyr1146 and either Tyr1150/1151, which are required for full InsR activation (34). In the absence of insulin, PTF treatment for 16 h did not increase the phosphorylation of InsR-β at Tyr1150/1151 compared with the control. In the presence of insulin, however, insulin increased InsR-β phosphorylation, and PTF pre-treatment for 16 h further enhanced insulin-induced phosphorylation by 28.93% (P<0.05) compared with insulin alone (Fig. 6A and B). These results suggest that PTF increases the phosphorylation of InsR-β in an insulin-dependent manner, and that PTF improves insulin-induced glucose uptake through another pathway independent of PI3K.

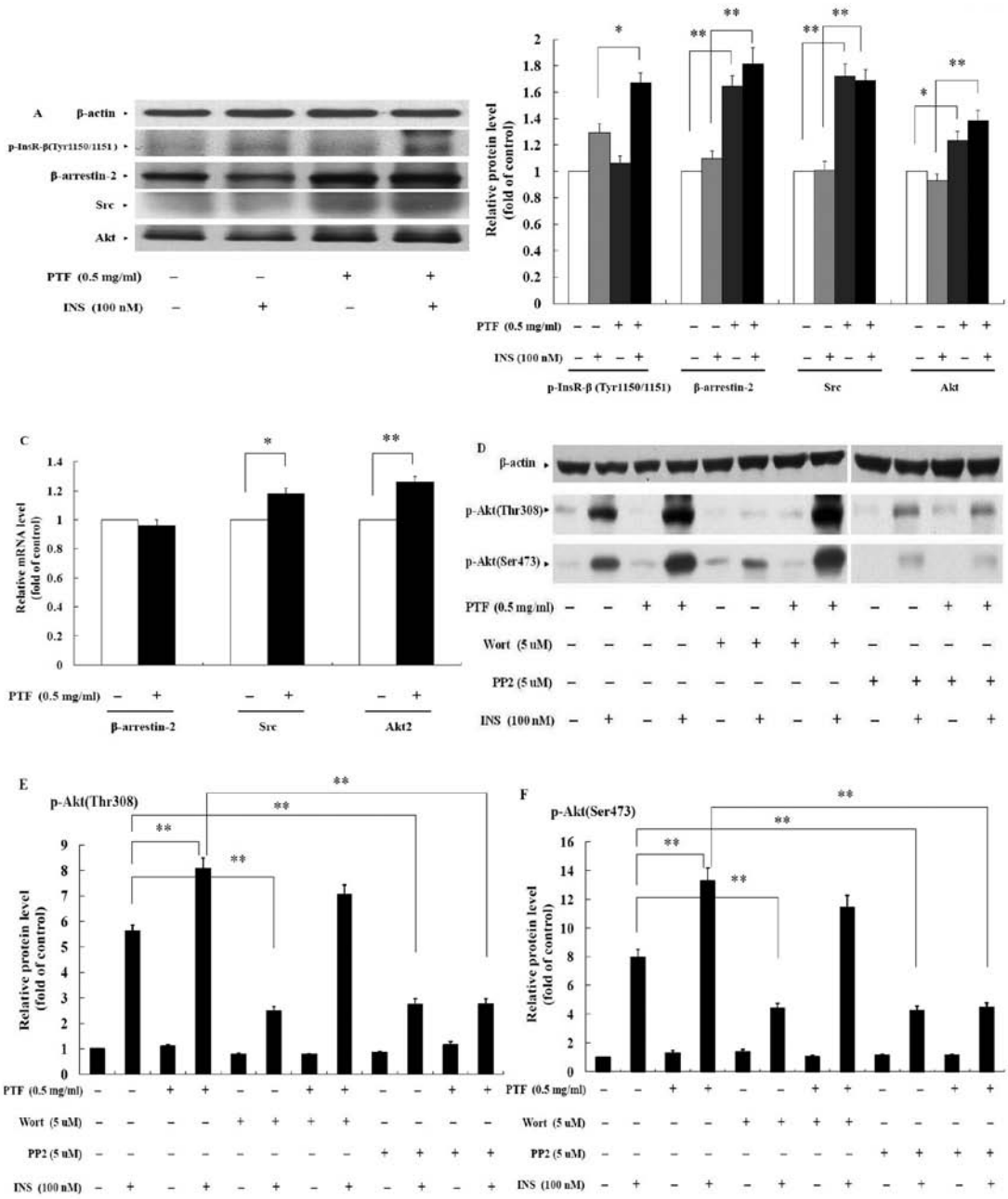

Figure 6. Effects of PTF on the β-arrestin-2-mediated signaling in C2C12 myotubes. C2C12 myotubes were pre-treated with or without PTF, wortmannin (Wort) or PP2 for 16 h after serum starvation in 1% BSA-DMEM, and the cells were then stimulated with or without insulin for 30 min. The cells were either lysed or used for total-RNA extraction. Whole cell lysate protein was examined by western blotting, and gene expression was assayed by real-time PCR. (A) Western blotting of p-InsR (Tyr1150/1151), β-arrestin-2, Src and Akt. (B) Densitometric analysis of active InsR (p-Tyr1150/1151), β-arrestin-2, Src and Akt. (C) Relative mRNA levels of β-arrestin-2, Src and Akt2. (D) Western blotting of p-Akt. (E) Densitometric analysis of active Akt (p-Thr308). (F) Densitometric analysis of active Akt (p-Ser473). *P<0.05, **P<0.01.

PTF enhances the protein expression of β-arrestin-2 with no effect on the basal gene expression in C2C12 myotubes. In addition to the PI3K pathway, the second pathway downstream of InsR is required for full Akt activation (12,15,16). In the β-arrestin-2-mediated signaling, β-arrestin-2 scaffolds Akt and Src to InsR upon insulin stimulation, causing the formation of the β-arrestin-2 signal complex, thereby regulating insulin-stimulated glucose disposal including glucose uptake. In view of the results, it was likely that PTF enhanced insulin-mediated glucose uptake via this signaling. Compared with the control and insulin alone, PTF pre-treatment of C2C12 myotubes for 16 h prominently increased the protein expression of β-arrestin-2 by 64.60% (P<0.01) and 65.51% (P<0.01) respectively (Fig. 6A and B). However, real-time PCR assay revealed that mRNA levels of β-arrestin-2 were not enhanced with PTF treatment for 16 h (Fig. 6C).

PTF increases the protein and the basal gene expression of Src in C2C12 myotubes. In the β-arrestin-2 complex (12,15,16), Src phosphorylates Akt on Tyr315/326 which is required for the subsequent phosphorylation of Akt at Thr308/Ser473. In this study, the protein expression of Src in C2C12 myotubes was elevated by 72.23% (P<0.01) and 67.48% (P<0.01) with PTF pre-treatment for 16 h compared with the control and

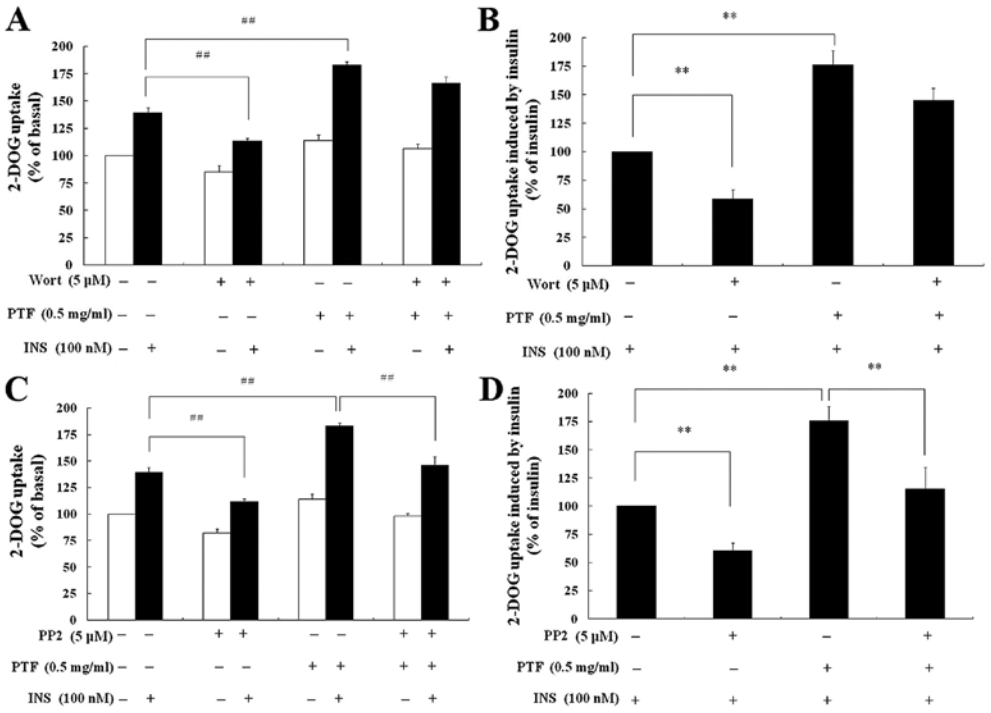

Figure 7. Improvement of insulin-induced glucose uptake by PTF is inhibited by PP2 but not wortmannin. Serum-starved C2C12 myotubes in 24-well culture plates were treated with wortmannin, PP2 and PTF for 16 h, and then stimulated with or without insulin for 20 min. Glucose uptake was determined with 2-DOG. (A) Effects of wortmannin on 2-DOG uptake. (B) Effects of wortmannin on PTF + insulin-induced 2-DOG uptake. (C) Effects of PP2 on 2-DOG uptake. (D) Effects of PP2 on PTF + insulin-induced 2-DOG uptake. n=6, **P<0.01, $^{\#\#}$P<0.01.

insulin alone, respectively (Fig. 6A and B). Moreover, PTF increased mRNA expression of Src compared with the control (P<0.05) (Fig. 6C).

PTF elevates the protein and the basal gene expression of Akt, and improves the phosphorylation of Akt in an insulin-dependent manner in C2C12 myotubes. Akt is the downstream target of Src in the β-arrestin-2 complex, and has three isoforms in mammals, each encoded by a different gene (Akt1-3), where Akt2 is closely related with the insulin-induced glucose uptake (35,36). Phosphorylation of Akt at Thr308/Ser473 stands for its full activation (6-8). In C2C12 myotubes, compared with the control and insulin alone, PTF pre-treatment for 16 h markedly increased the protein expression of Akt by 23.61% (P<0.05) and 48.83% (P<0.01), respectively (Fig. 6A and B). Similarly, mRNA levels of Akt2 with PTF treatment for 16 h were enhanced (P<0.01) compared with the control (Fig. 6C).

In the absence of insulin, PTF did not elevate the phosphorylation of Akt at Thr308 or Ser473. The phosphorylation was significantly increased with insulin stimulation for 30 min, and PTF further enhanced the insulin-induced phosphorylation at Thr308 by 44.02% (P<0.01) (Fig. 6D and E) and Ser473 by 66.10% (P<0.01) (Fig. 6D and F) after PTF pre-treatment for 16 h.

Wortmannin, an inhibitor of PI3K, used to pre-treat C2C12 myotubes for 16 h, remarkably inhibited insulin-induced phosphorylation of Akt at Thr308 by 55.95% (P<0.01) (Fig. 6D and E) and Ser473 by 44.56% (P<0.01) (Fig. 6D and F) compared with insulin alone, but failed to inhibit PTF-induced phosphorylation (P>0.05) (Fig. 6D, E and F).

Similarly, PP2, an inhibitor of Src, significantly reduced insulin-induced phosphorylation of Akt at Thr308 by 51.18% (P<0.01) (Fig. 6D and E) and Ser473 by 46.83% (P<0.01) (Fig. 6D and F) compared with insulin alone. Furthermore, PP2 also reduced PTF-induced phosphorylation at Thr308 by 65.74% (P<0.01) (Fig. 6D and E) and Ser473 by 66.06% (P<0.01) (Fig. 6D and F) compared with PTF + insulin.

Together, PTF increased the protein and the basal gene expression of Akt, and improved insulin-induced phosphorylation of Akt, which was inhibited by PP2 but not wortmannin. The data support the idea that PTF improves insulin-induced glucose uptake through the β-arrestin-2-mediated signaling in C2C12 myotubes.

Effects of wortmannin and PP2 on PTF-induced glucose uptake in C2C12 myotubes. Compared with insulin alone, wortmannin pre-treatment for 16 h resulted in a decrease in insulin-mediated 2-DOG uptake by 42.81% (P<0.01) (Fig. 7B), and PP2 by 38.33% (P<0.01) (Fig. 7D). Compared with PTF + insulin, wortmannin + PTF + insulin only inhibited insulin-induced 2-DOG uptake by 15.79% (P>0.05) (Fig. 7B), but PP2 by 35.29% (P<0.01) (Fig. 7D).

Discussion

Chinese herbs have been used to treat diabetes for thousands of years in Chinese clinical practice, and have become popular complementary and alternative medicine in the treatment of metabolic diseases by improving IR (37,38). It is worthwhile to screen insulin sensitizing reagents from Chinese herbs, and to elucidate their molecular mechanisms. In this study, we found that PTF increased insulin-stimulated glucose uptake in a dose-dependent manner, and prevented PA-induced IR in C2C12 myotubes, which conform to our previous report

(28). Insulin signaling mainly refers to two pathways: the Ras-mitogen-activated protein kinase (MAPK) pathway, and the PI3K signaling. The former regulates expression of some genes, and controls growth and differentiation of cells (5,6,8). The latter mediates most of the metabolic actions of insulin including insulin-induced glucose uptake. It is agreed that PA results in IR by restraining the PI3K pathway (39,40), and that enhancing the activity of the PI3K pathway is the key strategy for improving glucose uptake and IR. In order to determine whether PTF has some relationship with the PI3K pathway in improving insulin-induced glucose uptake, PI3K was hereby assayed, which consists of a regulatory (p85) and a catalytic subunit (p110) and plays a key role in the PI3K pathway. In the basal state, p85 binds to p110, thus inhibiting the activity of p110. Upon insulin stimulation, p85 associates with IRS and sets p110 free. In turn p110 catalyzes the production of PIP_3, which recruits PDK1/2 and Akt (8,41). In this study, PTF had no effects on the protein expression of p85 or the activity of PI3K. Interestingly, PTF prompted insulin-induced tyrosine phosphorylation of InsR-β at Tyr1150/1151, the feature of InsR standing for the earliest activation, which is required for full kinase activation of InsR in triggering insulin signaling. The results indicate that PTF increases insulin-induced glucose uptake dependent on InsR but not PI3K, suggesting that PTF improves insulin-stimulated glucose uptake through another pathway.

During 2009, Luan *et al* (12) found another novel insulin signaling involving β-arrestin-2, Src, InsR, and Akt, which provide new insight into the molecular pathogenesis of IR, and implicate a new strategy for screening insulin-sensitizing reagents to improve IR (42). Interestingly, PTF significantly enhanced the protein expression of β-arrestin-2 in C2C12 myotubes. It is generally accepted that β-arrestin-2 is an important adaptor in modulating the strength and duration of cellular signaling by scaffolding and interacting with a lot of cytoplasmic proteins including InsR, Src and Akt (15,16,43). Additionally, allowing for the improvement in the phosphorylation of InsR-β by PTF, we inferred that PTF had some relationship with the β-arrestin-2-mediated signaling. Western blotting and real-time PCR assay revealed that PTF not only increased the protein expression of Src and Akt, but also prompted the gene expression of Src and Akt. According to the novel signaling, β-arrestin-2 scaffolds Src and Akt to InsR upon insulin stimulation, causing the formation of the β-arrestin-2 signal complex. Following autophosphorylation of InsR-β, Src phosphorylates Akt on Tyr315/326, which enhances the phosphorylation of Akt at Thr308 by PDK1 and at Ser473 by PDK2 (5,6,8). To our surprise, PTF increased the phosphorylation of Akt at Thr308 and Ser473.

In order to further confirm the results, PP2, the inhibitor of Src, was used to inhibit the activity of the β-arrestin-2-mediated signaling. The results exhibited that PP2 not only inhibited the insulin-induced phosphorylation of Akt, but also depressed the PTF-induced phosphorylation of Akt. Together, PTF had beneficial effects on the β-arrestin-2-mediated signaling. Since, PTF had no effects on PI3K, wortmannin, the inhibitor of PI3K, was used to inhibit the activity of the PI3K pathway. The results showed that wortmannin inhibited insulin-induced phosphorylation of Akt, but did not depress PTF-induced phosphorylation. The present study does not prove that the PI3K pathway is not required for the PTF-induced increase in insulin-mediated phosphorylation of Akt, because the phosphorylation of Akt at Thr308 and Ser473 is dependent on PI3K, and Src only affects the phosphorylation at Thr308 and Ser473 via phosphorylating Akt on Tyr315/326. PTF had beneficial effects on the β-arrestin-2-mediated signaling, thus to some extent compensating for the loss of the phosphorylation of Akt by wortmannin. On the other hand, PTF contains a number of constituents including typhaneoside, isorhamnetin, isorhamnetin-3-O-neohesperidoside, kaempferol, quercetin and so on (21,44). It has been reported that isorhamnetin increases Akt activity in PC12 cells (45), kaempferol regulates lipopolysaccharide-induced phosphorylation of Akt in BV2 microglial cells (46), and quercetin protects oligodendrocyte precursor cells from oxygen/glucose deprivation injury *in vitro* via upregulating phosphorylation of Akt (47). It is expected that a certain ingredient or a specific constituent directly represses wortmannin, thus relieving the inhibition of wortmannin on the phosphorylation of Akt at Thr308/Ser473, which needs further studying. Activated Akt promotes insulin-stimulated translocation of Glut4 from intracellular organelles (endosomes) to cell surface within insulin-responsive tissues including muscle and fat, where Glut4 binds glucose and is in charge of glucose uptake (13). After transport of glucose into the skeletal muscle cells, glucose is mainly oxidized for energy, and used for glycogen synthesis. Skeletal muscle is not the site of gluconeogenesis due to a lack of gluconeogenesis enzyme. Consistent with our results involving Akt, glucose uptake induced by PTF was blocked by PP2 but not wortmannin, which further proved the beneficial effects of PTF on the β-arrestin-2-mediated signaling.

Furthermore, western blotting and real-time PCR analysis showed that PTF enhanced the protein expression of β-arrestin-2, but did not increase the gene expression. The ubiquitin system-induced ubiquitination and degradation of protein is an essential cellular mechanism. As previously reported (48-50), β-arrestin-2 is also an important regulator in the ubiquitin system-mediated ubiquitination of target proteins. β-arrestin-2 not only scaffolds and recruits ubiquitin ligase substrates, such as GPCRs, to the ubiquitin ligase Mdm2, thereby augmenting E3-mediated ubiquitination of target proteins and blocking relevant cellular signaling, but also interacts with Mdm2, causing β-arrestin-2 itself degradation by Mdm2 but competitively inhibiting ubiquitination of target proteins. Therefore, we propose that PTF may inhibit ubiquitination and degradation of β-arrestin-2 to enhance its protein levels, which merits further investigation.

In conclusion, PTF increases insulin-induced glucose uptake through the β-arrestin-2-mediated signaling in C2C12 myotubes. The findings suggest the potential uses of PTF, or compounds derived thereof, against type 2 diabetes and metabolic syndrome.

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