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EFFECTS OF *POLLEN TYPHAE* TOTAL FLAVONE ON β-ARRESTIN-2/SRC/ AKT SIGNALING IN ADIPOSE TISSUES OF TYPE 2 DIABETIC RATS

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Abstract

Background: *Pollen Typhae* total flavone (PTF), the extract from *Pollen Typhae*, a Chinese herbal medicine, has been reported to improve insulin resistance in type 2 diabetic rats, but the potential mechanisms keep unclear.

Materials and Methods: Type 2 diabetic model rats were induced by high-fat diet and low-dose streptozotocin, and then were administered PTF by intragastrical gavage. After treatment for 4 weeks, insulin receptor-β level in adipose tissues was determined by ELISA, and the protein expression was analyzed by western blotting.

Results: Administration of PTF increased insulin receptor-β level and enhanced β-arrestin-2 protein expression in adipose tissues of type 2 diabetic rats. Although having no effect on the protein expression of Src or Akt, PTF promoted phosphorylation of Src at Tyr416 and Akt at Ser473.

Conclusion: The results indicate that PTF has beneficial effects on the β-arrestin-2/Src/Akt signaling in adipose tissues of type 2 diabetic rats, implying the underlying mechanisms of PTF in ameliorating insulin resistance.

Key words: Chinese medicine, type 2 diabetes, signal transduction, insulin resistance, β-arrestin-2

Introduction

Pollen Typhae, the pollen of all species of *Typha* such as *T. angustifolia*, *T. latifolia*, *T. angustata* and *T. orientalis*, is a Chinese herbal medicine. *Pollen Typhae* has been widely used to treat type 2 diabetes and other diseases characterized by insulin resistance such as metabolic syndrome and non-alcohol fatty liver disease in China. We have ever reported that *Pollen Typhae* total flavone (PTF), the extract from *Pollen Typhae* which mainly contains typhaneoside and other constituents (Feng et al. 2012a), ameliorates insulin resistance in type 2 diabetic rats (Feng et al. 2014), but the potential mechanisms remain to be fully elucidated.

Insulin resistance, a hallmark of type 2 diabetes, is simply the defect of insulin action in triggering insulin signaling in peripheral insulin-sensitive tissues, including adipose tissues, liver, and skeletal muscles. In addition to phosphatidylinositol-3 kinase (PI3K) pathway, another insulin signaling has been recently discovered (Luan et al. 2009). Upon insulin stimulation, β-arrestin-2 - an important adaptor in mediating cellular signal pathway - scaffolds Src and Akt to upstream insulin receptor (InsR), thus promoting the formation of a β-arrestin-2 signal complex (Luan et al. 2009). In the complex Src phosphorylates Akt on Tyr315/326, which strengthens phosphorylation of Akt at Thr308/Ser473. Phosphorylated Akt mediates downstream kinases and transcription factors, thus regulating most of the metabolic actions of insulin (Jiang and Qiu 2003; Luan et al. 2009; Feng et al. 2011). Deficiency of β-arrestin-2 causes inability of the complex formation and dysfunction of the signaling, thereby resulting in insulin resistance. Overexpression of β-arrestin-2, however, promotes the formation of the

β-arrestin-2 complex, thus improving insulin resistance in animals (Luan et al. 2009). Inhibition of Src by PP2 leads to a reduction in phosphorylation of Akt at Thr308/Ser473 in C2C12 myotubes and Hep3B hepatocytes, thereby decreasing glucose uptake and insulin sensitivity (Luan et al. 2009; Feng et al. 2012b). It is well known that palmitate, a saturated fatty acid, contributes to insulin resistance involving complex mechanisms (Ishii et al. 2015). Moreover, although having no effect on the protein expression of β-arrestin-2, palmitate causes insulin resistance through, at least in part, down-regulating the phosphorylation of Src and Src-mediated phosphorylation of Akt in C2C12 myotubes (Feng et al. 2012b).

In order to reveal the underlying molecular mechanisms of PTF in improving insulin resistance, the study aimed to observe the effects of PTF on the β-arrestin-2/Src/Akt signaling in type 2 diabetic rats induced by high-fat diet and low-dose streptozotocin. The determination InsR-β level was also aimed at in the study.

Materials and Methods

Animal Model and Treatment

Male Sprague-Dawley rats (weight 160-200 g) were purchased from Hunan Slaccas Jingda Laboratory Animal Company Limited (Changsha, China). Normal pellet diet with 4.6% fat (about 10% of calories as fat) and high fat diet (about 40% of calories as fat) were provided by Shanghai Slaccas Laboratory Animal Company Limited (Shanghai, China). Type 2 diabetic model rats were induced by high-fat diet and low-dose streptozotocin (35 mg/kg, i.p.) in line with our previous description (Feng et al. 2014). The model rats were randomly assigned to 3 subgroups: the model group (MOD, n = 8), the *Pollen Typhae* total flavone group (PTF, n = 9), and the rosiglitazone group (ROS, n = 8). The control group (CON, n = 9; non-diabetic rats) for type 2 diabetic rats was set up. The rats in the PTF group and the rosiglitazone group were given 200 mg/kg·d PTF (extracted from the pollen of T. angustifolia) and 4 mg/kg·d rosiglitazone (dissolved in purified water) by intragastric administration, respectively; while the rats in the control group and the model group received an equal volume of purified water per day, respectively. After treatment for 4 weeks, the rats were anesthetized with pentobarbital sodium (40 mg/kg, i.p.), and visceral adipose tissues in each group were collected and kept in -80°C fridge. Then, relative indexes were checked. All experimental procedures were in accordance with the internationally accepted principles for laboratory animal use and care. The study was approved by the Ethics Committee of Guangxi University of Chinese Medicine.

Reagents

Antibodies directed against β-arrestin-2, Akt (total), Akt (phosphorylated Thr473), and Src (phosphorylated Tyr416) were purchased from Cell Signaling Technology (Danvers, MA, USA). Antibody to Src was obtained from Epitomics (Burlingame, CA, USA). Secondary horseradish peroxidase (HRP)-conjugated antibodies and streptozotocin were from Sigma (St. Louis, MO, USA). ECL Western Blotting Substrate was acquired from Pierce (Rockford, IL, USA). Rat InsR-β ELISA Kit was from Bio-Swamp Life Science (Shanghai, China). PTF was provided and determined by Xi'an Salao Bio-Technology Company Limited (Xi'an China). Rosiglitazone was from Glaxo Company Limited (Tianjin, China). Protein assay kit, phosphate buffered saline (PBS), and RIPA lysis buffer for western blot were obtained from Beyotime Institute of Biotechnology Co., Ltd. (Shanghai, China).

Determination of InsR-β

Adipose tissues was homogenized with PBS, and subsequently centrifuged to remove the tissue debris at 3,000 rpm for 5 min at 4°C. The protein concentration of supernatant was analyzed using a BCA protein assay kit. Then, InsR-β was measured by ELISA per the manufacturer's instructions.

Western Blot Analysis

The protein expression in adipose tissues was analyzed by western blotting according to a previously described procedure with some

modifications (Feng et al. 2012a). Briefly, tissue lysate was prepared using RIPA lysis buffer, and supernatant was collected after centrifugation (12,000 rpm for 5 min, 4°C). Then, the protein concentration of each sample supernatant was determined using protein assay kit. The protein was separated via SDS-PAGE and electrophoretically transferred to polyvinylidene difluoride membranes (Millipore, Billerica, MA, USA). The membranes were incubated with the primary antibodies overnight at 4 °C and then with the appropriate secondary HRP-conjugated antibodies for 1-2 h at room temperature. The targeted proteins were visualized by incubation with Pierce ECL Western Blotting Substrate.

Results and Discussion

InsR is comprised of two intracellular β subunits and two extracellular α subunits - β subunits are auto-phosphorylated on tyrosine upon insulin binding to InsR. Recent study reported that the protein expression of InsR-β was significantly decreased in skeletal muscle of type 2 diabetic rats (Dong et al. 2013). In this study, InsR-β level was also, to some extent, reduced in adipose tissues of type 2 diabetic rats and administration of PTF significantly increased InsR-β level (Fig. 1). Furthermore, we have ever reported that PTF promotes phosphorylation of InsR-β in an insulin-dependent manner in C2C12 myotubes *in vitro* (Feng et al. 2012a). Although, β-arrestin-2 level in the model group was not markedly reduced when compared with the control group, PTF treatment significantly increased β-arrestin-2 protein expression in the rats' adipose tissues when compared with the model group (Fig. 2). According to the past reports (Luan et al. 2009; Feng et al. 2012b), β-arrestin-2 protein expression is not changed in adipose tissues of high-fat diet-induced insulin-resistant mice and palmitate-induced insulin-resistant skeletal muscle cells, which is consistent with this study. Because of the key role of β-arrestin-2 in regulating the insulin signaling and an increase in β-arrestin-2 protein expression by PTF, it is likely that PTF has beneficial effects on the β-arrestin-2/Src/Akt signaling.

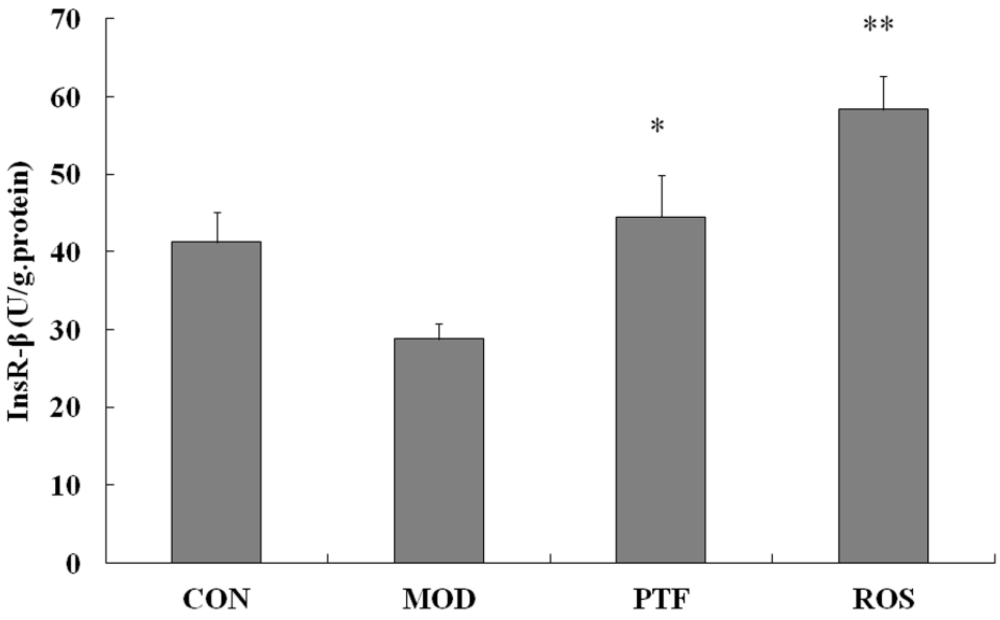

Figure 1: Effect of PTF on InsR-β level in adipose tissues of type 2 diabetes rats. After treatment, InsR-β level in adipose tissues was determined using ELISA. The data are presented as means ± standard error (SE). The significance among multiple groups was analyzed by one-way analysis of variance (ANOVA) followed by LSD test. $P < 0.05$ was considered to be statistically significant. $^{*}P < 0.05$, $^{**}P < 0.01$ *vs*. model group (MOD).

It is confirmed that Akt phosphorylation at Thr308/Ser473 is significantly decreased in insulin-targeted organs of type 2 diabetic animals, while total Akt protein expression keeps no changes or is decreased (Yuan et al. 2007; Gandhi et al. 2013). As a protein tyrosine kinase, the activity of Src is regulated by tyrosine phosphorylation. Phosphorylation of Src at Tyr527 means less activity, while phosphorylation at Tyr416 increases the enzyme activity (Hunter 1987). Src can be directly associated with Akt through the interaction between its SH3 domain and a conserved proline-rich motif (PXXP) in the C-terminal regulatory region of Akt, and induces phosphorylation of Tyr315 in Akt, thus promoting Akt phosphorylation at Thr308/Ser473 (Jiang and Qiu 2003; Lodeiro et al. 2009). Therefore, we analyzed the effects of PTF on the protein

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expression of Src and Akt. In this study, the protein expression of Src or Akt was not remarkably changed in the model group when compared with the control group, and administration of PTF did not increase the protein expression. The phosphorylation of Src at Tyr416 and Akt at Ser473, however, was markedly decreased in the model group rats, and PTF treatment significantly enhanced the phosphorylation (Fig. 2). In palmitate-induced insulin-resistant C2C12 myotubes, insulin-stimulated phosphorylation of Src at Tyr416 and Akt at Thr308/Ser473 is remarkably reduced, with no changes in their total protein expression (Feng et al. 2012b). And in aortic strips of type 2 diabetic rats, the phosphorylation of Src and Akt dependent of insulin triggering is also decreased (Nemoto et al. 2011). These were all consistent with this study. It is well known that rosiglitazone, an insulin sensitizer, ameliorates insulin resistance by activating peroxisome proliferator-activated receptors-γ, thereby enhancing the activity of the PI3K pathway, an important insulin signal pathway. In the study, rosiglitazone was taken as a control for PTF, and enhanced InsR-β level and Akt phosphorylation when compared with the model group, but could not increase β-arrestin-2 protein expression or Src phosphorylation (Fig. 2), which was different from PTF.

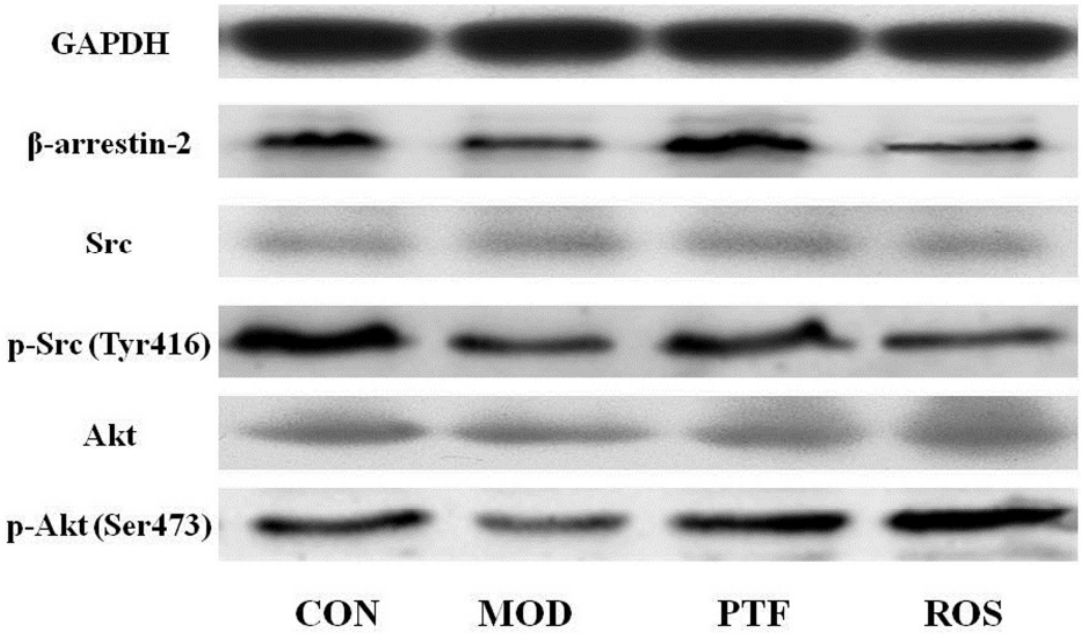

Figure 2: Effects of PTF on protein expression of β-arrestin-2, Src, and Akt as well as their phosphorylation in adipose tissues of type 2 diabetic rats.

After treatment, the protein expression in adipose tissues was analyzed by western blotting as materials and methods described.

Conclusion

Type 2 diabetes often coexists with obesity, with more adipose. Adipose tissues, the key insulin targeted tissues, play an important role in maintaining insulin-mediated metabolic action, including glucose uptake and disposal as well as fat mobilization (He et al. 2006; Mitrou et al. 2009). Dysfunction of insulin pathway in adipose tissues causes insulin resistance. PTF promotes glucose uptake and consumption in 3T3-L1 adipocytes and C2C12 myotubes *in vitro* (He et al. 2006; Feng et al. 2012a), suggesting the potential anti-insulin resistant action of PTF. *In vivo*, PTF improves insulin resistance in type 2 diabetic rats induced by high-fat diet and low-dose streptozotocin (Feng et al. 2014). Additionally, PTF can not affect PI3K activity in C2C12 myotubes (Feng et al. 2012a), a critical node in PI3K pathway. Here, PTF increased InsR-β level, enhanced β-arrestin-2 protein expression, and promoted phosphorylation of Src and Akt in adipose tissues of type 2 diabetic rats, implying the beneficial effects of PTF on the β-arrestin-2/Src/Akt signaling. Together, PTF ameliorates insulin resistance through, at least in part, the β-arrestin-2/Src/Akt signaling in adipose tissues of type 2 diabetic rats.

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Conflict of Interest: No potential conflict of interest relevant to this article was reported.

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