doi.org/10.36721/PJPS.2022.35.6.REG.1669-1676.1

Pollen typhae total flavone suppresses A7r5 cell proliferation promoted by insulin involving the MEK1/2-ERK1/2 cascades

Xiao-Tao Feng[1,2]*#, Hui-Ming Duan[2#] and Ruyan Wen[2#]
[1]Guangxi Key Laboratory of Chinese Medicine Foundation Research, Guangxi University of Chinese Medicine, Nanning, China
[2]Guangxi Scientific Experimental Center of Traditional Chinese Medicine, Guangxi University of Chinese Medicine, Nanning, China

Abstract: *Pollen typhae*, a traditional medicine in China, performs an anti-diabetic function and has anti-atherosclerosis effects involving suppression of vascular smooth muscle cell proliferation. However, the potential mechanisms keep to be revealed. The present study intended to investigate the influences of *Pollen typhae* extract named *Pollen typhae* total flavone (PTF) on A7r5 cell proliferation promoted by insulin and to uncover the underlying mechanisms. Proliferation and viability were evaluated by CCK-8 method. Western blotting was adopted to analyze the protein expression. Insulin promoted A7r5 cell proliferation, while PTF suppressed insulin-promoted proliferation in a concentration-dependent fashion. Although PTF did not change c-Jun N-terminal kinase (JNK), p38 mitogen-activated protein kinase (p38MAPK) or MAPK kinase 1/2 (MEK1/2) protein expression and failed to affect the phosphorylation of JNK and p38MAPK, PTF remarkably inhibited extracellular signal-regulated kinase 1 and 2 (ERK1/2) protein expression and reduced ERK1/2 and MEK1/2 phosphorylation in A7r5 cells stimulated by insulin. Insulin-induced proliferation of A7r5 cells was abolished by inhibiting ERK1/2, which was in line with PTF. These findings indicate that PTF suppresses insulin-promoted proliferation of A7r5 cells involving the MEK1/2-ERK1/2 cascades, providing new insight into the potential uses of PTF for treatment of diabetic atherosclerosis.

Keywords: Chinese medicine, vascular smooth muscle cells, proliferation, insulin resistance, signaling pathway.

INTRODUCTION

Type 2 diabetic patients often bear a higher risk of macrovascular disease than non-diabetic ones (Hayfron-Benjamin *et al.*, 2021), such as cardiovascular disease, cerebrovascular disease and peripheral artery disease. It is generally accepted that atherosclerosis, characterized by atherosclerotic plaque, is a foundational contributor to the development of diabetic macrovascular complications (Li *et al.*, 2016) and partly results from excessive proliferation and migration of vascular smooth muscle cells (VSMCs). Systemic insulin resistance, a pathophysiological characteristic of type 2 diabetes, often coexists with hyperinsulinemia. Indeed, insulin enhances VSMC proliferation and migration (Zhang *et al.*, 2021; Peng *et al.*, 2021). Studies indicated that mitogen-activated protein kinase (MAPK) signaling pathway is initiated when insulin binds to insulin receptor (InsR), thereby regulating proliferation and migration of VSMCs to accelerate atherosclerosis (Fu *et al.*, 2021). Moreover, VSMCs synthesize and secrete extracellular matrix, which associate with vascular remodeling and restenosis and the development of atherosclerosis. Insulin enhances synthesis and secretion of extracellular matrix from VSMCs including collagen and elastin, thus leading to rearrangements of extracellular matrix and deteriorating atherosclerosis (Ruiz-Torres *et al.*, 1998; Shi *et al.*, 2012). Therefore, it is a promising strategy to prevent and cure type 2 diabetic atherosclerosis and macro vascular complications by inhibiting insulin-promoted proliferation of VSMCs.

Pollen Typhae, a traditional medicine in China, performs various functions including improving circulation, anti-inflammation, anti-oxidation and analgesic effect (Chen *et al.*, 2017; Zeng *et al.*, 2020; Chen *et al.*, 2021). Studies revealed that *Pollen typhae* contains multiple flavonoids including typhaneoside, quercetin, isorhamnetin-3-O-neohesperidoside, naringenin, kaempferol and isorhamnetin (Chen *et al.*, 2017; Zeng *et al.*, 2020; Gao *et al.*, 2021). We have ever reported the anti-diabetic activity of *Pollen typhae* total flavone (PTF) (Feng *et al.*, 2014), which was extracted from *Pollen typhae* and mainly compose of typhaneoside as well as other ingredients (Feng *et al.*, 2012). PTF reduces blood glucose, improves dyslipidemia and increased systemic insulin sensitivity in an animal model of type 2 diabetes (Feng *et al.*, 2014). Further study showed that PTF promotes glucose uptake in an insulin-dependent manner and guards against insulin resistance triggered by palmitic acid (PA) in skeletal muscle cells (Feng *et al.*, 2012), which are due to the beneficial role of PTF in affecting the β-arrestin-2-dependent signaling pathway (Feng *et al.*, 2012; Feng *et al.*, 2015). It is a known fact that PA impairs glucose-stimulated insulin secretion (GSIS) function of insulin producing cells, while PTF protects against PA-induced impairment of GSIS through G-protein-coupled receptor 40 (GPR40) signaling pathway (Feng *et al.*, 2017). Lei *et al*. (2018) reported that Typhae pollen polysaccharides decrease circulating TNF-α and IL-6 levels, reduce the expression of basic fibroblast growth factor and vascular endothelial growth factor in retinal tissues and improve retinal injury in a rat model of

**Corresponding author:* e-mail: fengxt2008@163.com
#*Contributed equally*

diabetes. Recently, *Pollen typhae* has been confirmed to inhibit VSMC proliferation (Zhao *et al.*, 1990; Nhiem *et al.*, 2010). Moreover, PTF attenuates oxidized low-density lipoprotein-induced endoplasmic reticulum stress in human VSMCs, thus relieving apoptosis (Chen *et al.*, 2019). These studies suggest the anti-atherosclerosis effects of *Pollen typhae*, but the underlying mechanisms remain to be demonstrated. Since PTF improves insulin resistance and *Pollen typhae* inhibits VSMC proliferation, we inferred that PTF may inhibit VSMC proliferation promoted by insulin, indicating the anti-atherosclerosis action of PTF for type 2 diabetes. In the present study, we intended to investigate the influence of PTF on insulin-stimulated proliferation of A7r5 rat aortic smooth muscle cells and to reveal the underlying mechanisms, which would display new enlightenment about the potential uses of PTF for the treatment of type 2 diabetic atherosclerosis.

MATERIALS AND METHODS

Reagents

Thermo Fisher Scientific (Grand Island, USA) provided fetal bovine serum (FBS), Dulbecco's modified Eagle's medium (DMEM) and BCA Protein Assay kits. Insulin solution, bovine serum albumin (BSA) and PD98059 were purchased from Sigma (St. Louis, USA). Corning, Inc. (New York, USA) offered cell culture plates. PTF was obtained and authenticated by Xi'an Salao Bio-Technology Co., Ltd. (Xi'an, China). The CCK-8 assay kits were from Dojingdo Molecular Technologies, Inc. (Rockville, USA). Beyotime Institute of Biotechnology (Shanghai, China) supplied RIPA cell lysis buffer. Primary antibodies targeted for extracellular signal-regulated kinase 1 and 2 (ERK1/2), c-Jun N-terminal kinase (JNK), MAPK kinase 1/2 (MEK1/2), Akt and their phosphorylated ones, phosphorylated p38MAPK (p-p38MAPK), phosphatidylinositol-3 kinase (PI3K)-p85 as well as GAPDH were provided by Cell Signaling Technology (Danvers, USA). Anti-p38MAPK antibody was supplied by Abcam (Cambridge, USA). Secondary antibody directed against rabbit and enhanced chemiluminescence (ECL) kits were purchased from Jackson Immuno research (West Grove, USA) and Shanghai Yi Sheng Biotechnology Co., Ltd. (Shanghai, China), respectively. Polyvinylidene difluoride membranes were acquired from Merck Millipore Ltd. (Billerica, USA).

Cell culture

The Type Culture Collection of the Chinese Academy of Science (Shanghai, China) offered A7r5 cells. This cells were growth in DMEM supplemented with 10% FBS under a humidified environment at 37°C with 7.5% CO2 atmosphere. DMEM was replaced every 48 h.

Proliferation evaluation

CCK-8 method was used to evaluate proliferation according to previous procedure with some modification (Duan *et al.*, 2021). In brief, A7r5 cells were cultured in high glucose DMEM with 10% FBS at an inoculation concentration of $2.5\sim4.0\times10^4$/ml. After 16~24h of inoculation, the medium was substituted with DMEM containing 1% FBS. After starvation overnight, the cells were intervened with insulin or/and PTF, PD98059 for 24, 48 or 72h, respectively. The medium was discarded and serum-free DMEM as well as CCK-8 solution were added to the cells. The cells were incubated for 1h and then checked the optical absorbance at 450nm using a micro plate reader (TECAN, Infinite® 200 Pro Nano Quant).

Viability assay

Viability was checked by CCK-8 method as previous procedure with certain modification (Liu *et al.*, 2021). A7r5 cells were seeded and grown confluence in culture plate 96 and then exposed to PTF (0, 0.0075, 0.015, 0.03, 0.06, 0.125, 0.25, 0.5, 1.0mg/ml) for 24, 48 and 72h, respectively. PTF was diluted with serum-free DMEM supplemented with 0.5% BSA. The medium was removed and the solution containing serum-free DMEM and CCK-8 was added. After incubation for 1 h, micro plate reader (TECAN, Infinite® 200 Pro Nano Quant) was used to check the optical absorbance at 450 nm.

Western blotting

The protein expression was detected using western blotting following previous operating process with appropriate modification (Feng *et al.*, 2017). A7r5 cells were treated with insulin or/and PTF for 24h and then rinsed with cold PBS. The cells were harvested and lysed to extract total protein in cold RIPA cell lysis buffer following ultrasonic lysis. The protein solution was centrifuged to discard the sediment and then checked the concentrations using a BCA Protein Assay kit according to the manufacturer's specifications. After heat denaturation and subsequent separation using 10% sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), the proteins were electro transferred to polyvinylidene fluoride (PVDF) membranes, which were then sealed with 5% fatty-free milk for 1h at 37°C. The membranes were probed individually with the appropriate primary antibodies to pass the night at 4°C. Following washing with TBS Tween buffer three times at room temperature, the membranes were labeled with secondary antibody directed against rabbit for 1h at room temperature. Ultimately, the membranes were exposed to ECL reagents to display the protein blots.

STATISTICAL ANALYSIS

All data from this study were statistically analyzed with SPSS 16.0 for Windows and the final results are expressed as means ±standard deviation (SD). To evaluate the dramatic differences between more than two groups, one-way analysis of variance (ANOVA) and subsequent

LSD test were used to perform the comparison. A value of P<0.05 was considered as statistically significant.

RESULTS

Insulin promotes proliferation of A7r5 cells

Compared with the control (0μM insulin), insulin at the concentration of 0.01~10μM significantly enhanced absorbance of A7r5 cells in a concentration-dependent fashion (all P<0.01) when the cells received treatment with insulin for 24 (fig. 1A) or 48h (fig. 1B). Moreover, the absorbance was also dramatically increased (all P<0.01) in an insulin dose-sensitive manner when the cells were incubated with insulin (0.1~10μM) for 72h (Fig. 1C). Together, insulin promotes A7r5 cell proliferation in a concentration- and time-dependent fashion.

PTF suppresses A7r5 cell proliferation promoted by insulin

After A7r5 cells exposure to insulin for 24, 48 or 72h (fig. 2) respectively, insulin at the concentration of 1μM significantly potentiated the absorbance of A7r5 cells (all P<0.01) in comparison with the control (free insulin). While the absorbance of the cells induced by insulin was obviously inhibited by PTF (0.06~0.5 mg/ml) in a dose-dependent fashion (P<0.05 or P<0.01) after treatment for 24h (fig. 2A) when compared with insulin (1.0 μM). In the same way, administration of PTF at the concentration of 0.03~0.5mg/ml for 48h (fig. 2B) and at the concentration of 0.125~0.5mg/ml for 72h (fig. 2C) also remarkably reduced insulin-induced absorbance of A7r5 cells in a concentration-dependent fashion (all P<0.01), respectively. Moreover, treatment with PTF at the concentration of 0.0075~1.0 mg/ml for 24, 48 or 72h (fig. 3) did not impair the viability of A7r5 cells in comparison with the control (free PTF). Additionally, intervention with PTF at 0.5mg/ml for 24h (fig. 4) markedly suppressed various dose (0.1~10μM) insulin-induced absorbance (all P < 0.01) and number of A7r5 cells (all P<0.05). After treatment for 24 or 48h (fig. 5), PTF decreased 1% FBS-induced absorbance of A7r5 cells in a concentration-dependent fashion in comparison with 1% FBS, respectively. The results indicate that PTF suppresses A7r5 cell proliferation promoted by insulin.

PTF regulates MAPK signaling pathway in A7r5 cells

To elucidate the underlying mechanisms of PTF inhibiting proliferation of A7r5 cells, it is necessary to investigate the influences of PTF on the MAPK signaling pathway. As shown in fig. 6, comparison with the control (free insulin), although insulin did not affect the protein expression of JNK, p38MAPK or their phosphorylation (fig. 6A, C and D), insulin dramatically promoted the protein expression and phosphorylation of ERK1/2 (fig. 6A and B, P<0.05 or P<0.01). Moreover, insulin markedly enhanced the phosphorylation of MEK1/2 (P<0.05), but failed to alter its protein expression (fig. 6E and F). Administration of PTF remarkably inhibited the protein expression of ERK1/2 (fig. 6A and B) and suppressed the phosphorylation of ERK1/2 as well as MEK1/2 (fig. 6E and F) when compared with insulin (P<0.05 or P<0.01). However, PTF did not affect the protein expression of JNK, p38MAPK or MEK1/2 (fig. 6A and C-F) and could not change the phosphorylation of JNK or p38MAPK (fig. 6A, C and D). Additionally, A7r5 cell proliferation promoted by insulin was abolished by PD98059 (fig. 6G), an inhibitor of ERK1/2.

Effects of PTF on PI3K/Akt signaling pathway in A7r5 cells

Fig. 7 showed that insulin did not significantly regulate the protein expression of PI3K-p85 or Akt in A7r5 cells, but apparently increased Akt phosphorylation (P<0.01) in comparison with the control (free insulin). In line with insulin, PTF was unable to change PI3K-p85 or Akt protein expression, but further enhanced insulin-induced phosphorylation of Akt (P<0.05) when compared with insulin.

DISCUSSION

Hyperinsulinemia accelerates atherosclerosis of type 2 diabetes, associating with increased proliferation and migration of VSMCs (Li *et al.*, 2015; Peng *et al.*, 2021). Increased VSMCs not only thicken arteries but also synthesize and secrete extracellular matrix, thus leading to vascular remodeling, restenosis and the development of atherosclerosis (Zeadin *et al.*, 2013; Li *et al.*, 2015). Indeed, chronic high-dose insulin therapy promotes proliferation of VSMCs and vascular remodeling (Li *et al.*, 2015), which is beneficial for the formation of atherosclerotic plaques. In the current study, insulin enhanced A7r5 cell proliferation in concentration-dependent fashion *In Vitro*, which was agreed with the past reports (Zhang *et al.*, 2021; Peng *et al.*, 2021). Administation of PTF abolished A7r5 cell proliferation promoted by insulin in a concentration-dependent manner. Moreover, PTF still restrained 1% FBS-evoked proliferation of A7r5 cells. Studies showed that the major flavonoid components of *Pollen typhae* such as naringenin and quercetin inhibit proliferation of VSMCs (Alcocer *et al.*, 2002; He *et al.*, 2022). Additionally, Zhu *et al*. (2019) reported that typhaneoside suppresses proliferation of acute myeloid leukemia (AML) cells. And kaempferol restrains KBM7R cells proliferation (Li *et al.*, 2022). Moreover, quercetin still represses growth of melanoma B16 cells (Qiang *et al.*, 2021). The results are in keeping with this study, suggesting the anti-atherosclerotic effects of PTF through inhibiting VSMC proliferation.

It is well known that insulin, a growth factor, controls multiple functions by initiating InsR and subsequent

signaling cascades, containing at least the MAPK and the PI3K/Akt signaling pathways (Taniguchi *et al.*, 2006; Fu *et al.*, 2021). In the present of insulin, InsR is autophosphorylated and then phosphorylates InsR substrate proteins (IRS). IRS binds to growth-factor receptor bound protein-2 (Grb2) through tyrosine residue motif, thus promoting Grb2 to connect son-of-sevenless (SOS) upon SH3 domain, which causes the activation of Ras. Activated Ras mediates activity of Raf and subsequent MAPK cascades. Moreover, phosphorylated InsR promotes Shc phosphorylation at tyrosine and then activates Ras through promoting interaction between Grb2 and SOS, thus triggering the MAPK cascades (Taniguchi *et al.*, 2006). MAPK contains multiple family members, including ERK1/2, p38MAPK and JNK. The MAPK signaling pathway is mainly responsible for the cell growth, proliferation, cycle, survival and differentiation. Additionally, activated IRS enhances the activity of PI3K and downstream Akt phosphorylation at Thr308 and Ser473, which regulates downstream targeted proteins including inhibition of glycogen synthase kinase 3 (GSK3) activity and forkhead box O1 (Foxo1) expression, promotion of glucose transporter-4 (GLUT4) translocation and rise of mTOR phosphorylation, thereby mediating quite a number of insulin functions involving metabolism, such as glycogen synthesis, glucose uptake, gluconeogenesis, protein synthesis and fat metabolism (Taniguchi *et al.*, 2006; de Campos Zani *et al.*, 2022). In the present study, although insulin could not change the protein expression of JNK or p38MAPK or their phosphorylation levels, insulin increased phosphorylation of MEK1/2 and enhanced ERK1/2 protein expression and phosphorylation, a downstream targeted protein of MEK1/2, suggesting the activation of the MAPK cascades. Interestingly, PTF was unable to change the protein expression or phosphorylation of JNK or p38MAPK, but significantly inhibited phosphorylation of MEK1/2 and suppressed ERK1/2 protein expression and phosphorylation, implying inactivation of the MEK1/2-ERK1/2 cascades. Moreover, PD98059, a specific inhibitor for ERK1/2 activity, abolished A7r5 cell proliferation promoted by insulin, which was in accordance with PTF. In fact, inhibition of the MAPK signaling pathway reverses high glucose-induced proliferation of VSMCs (Wang *et al.*, 2021). Typhaneoside, one of the main flavones from the *Pollen typhae* extract, makes AML cells arrest at G2/M phase (Zhu *et al.*, 2019) and inhibits glutamate release from rat cerebrocortical nerve through inactivation of ERK1/2-dependent cascade but not JNK or p38MAPK (Chiu *et al.*, 2021). Naringenin, another flavonoid from the *Pollen typhae* extract, inhibits ERK1/2 phosphorylation but not JNK or p38MAPK phosphorylation, thus blocking TNF-α-induced VSMC proliferation (Chen *et al.*, 2012). Quercetin attenuates TGF-β1-induced proliferation of pulmonary arterial endothelial cells and causes growth inhibition of breast cancer cell line involving dephosphorylation of ERK1/2 (Huang *et al.*, 2017; Safi *et al.*, 2021). Moreover, quercetin induces apoptosis of VSMCs by activating adenosine 5‘-monophosphate-activated protein kinase (AMPK) pathway (Kim *et al.*, 2020). In view of the regulatory effect of the MAPK signaling pathway on cell proliferation, it is likely that PTF inhibits insulin-induced proliferation of A7r5 cells through, at least in part, the MEK1/2-ERK1/2 cascades.

In the present study, PTF further increased Akt phosphorylation in A7r5 cells promoted by insulin, but was unable to alter the protein expression of p85, a regulatory subunit of PI3K. Our previous study indicated that PTF enhances insulin-stimulated glucose uptake as well as phosphorylation of Akt in skeletal muscle cells, but fails to change p85 protein expression and PI3K activity, which are linked to the β-arrestin-2-dependent pathway (Feng *et al.*, 2012). Moreover, PTF improves glycolipid metabolism and ameliorates insulin resistance through the β-arrestin-2/Src/Akt pathway in adipose tissues of a rat model of type 2 diabetes (Feng *et al.*, 2014; Feng *et al.*, 2015). According to the reports, naringenin downregulates high glucose- or TNF-α-induced VSMC proliferation through interfering the PI3K/Akt signaling pathway (Chen *et al*. 2012; He *et al.*, 2022). Similarly, quercetin decreases phosphorylation of Akt, suppresses TGF-β1-induced proliferation of pulmonary arterial endothelial cells (Huang *et al.*, 2017) and exerts the anticancer efficacy for breast cancer (Safi *et al.*, 2021). Indeed, the PI3K/Akt signaling pathway still controls insulin mitogenic actions, regulates cell growth and proliferation, differentiation, cell cycle and mediates apoptosis as well as autophagy (Duan *et al.*, 2021), but the regulation of metabolic actions is the main function of this insulin pathway. In this study, PTF had no effect to control p85 protein expression in A7r5 cells. Moreover, PTF improves metabolic actions of insulin through β-arrestin-2-mediated Akt phosphorylation but not PI3K-dependent Akt phosphorylation (Feng *et al.*, 2012; Feng *et al.*, 2014; Feng *et al.*, 2015). Study also reported that kaempferide ameliorates glycolipid metabolism disorder in high-fat diet mouse model via triggering the PI3K/Akt signaling pathway (Tang *et al.*, 2021). Moreover, kaempferide limits myocardial injury induced by ischemia/reperfusion which relates to the PI3K/Akt-mediated GSK-3β activation (Wang *et al.*, 2017). And typhaneoside can improve cardiac morphological structure through controlling autophagy by enhancing phosphorylation of Akt and mTOR (Zhang *et al.*, 2020). These results are in line with this study.

Additionally, PTF ameliorates systemic insulin sensitivity *In Vivo*, thus decreasing serum insulin levels of type 2 diabetic rats (Feng *et al.*, 2014) and insulin-induced proliferation of VSMCs. In addition to the improvement of the insulin signaling pathway (Feng *et al.*, 2012; Feng *et al.*, 2015), PTF ameliorates inflammation to increase

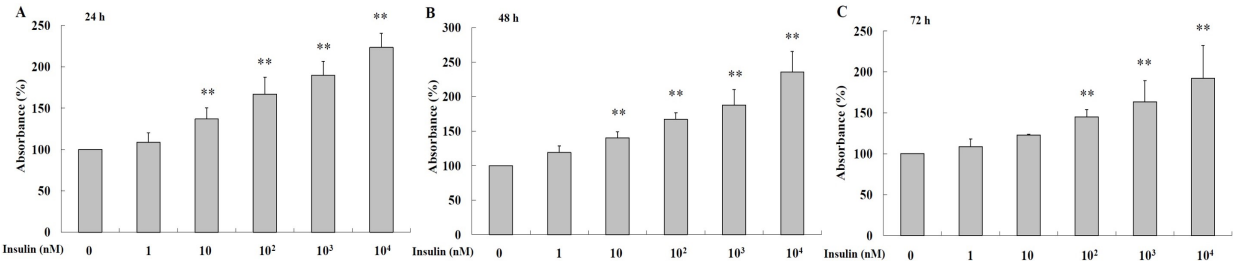

Fig. 1: Effects of insulin on the proliferation of A7r5 cells. After treatment with insulin for 24 (A), 48 (B) and 72h (C), respectively, the proliferation of A7r5 cells was analyzed by CCK-8 method. **P<0.01 vs. the control (0.0nM insulin). n=4.

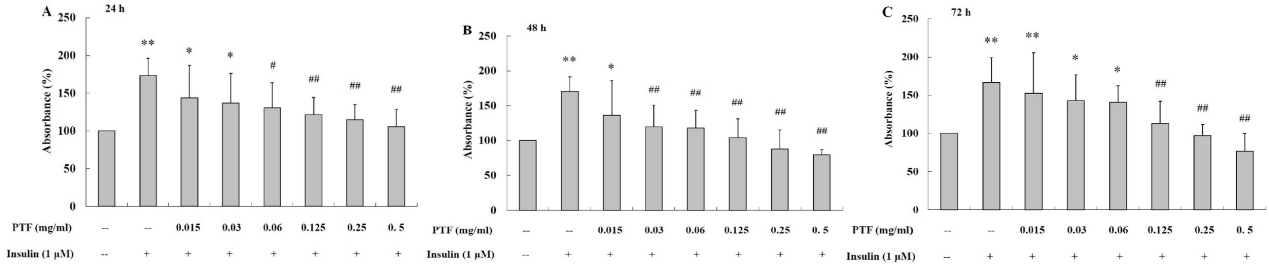

Fig. 2: Effects of PTF on insulin-induced proliferation of A7r5 cells. After treatment with insulin or/and PTF for 24 (A), 48 (B) and 72h (C), respectively, the proliferation of A7r5 cells was analyzed by CCK-8 method. *P<0.05, **P<0.01 vs. the control (free insulin); $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. 1μM insulin. n=5.

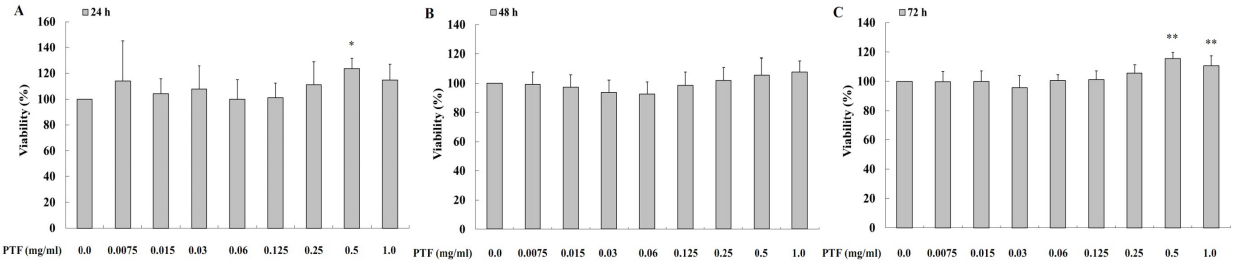

Fig. 3: Effects of PTF on the viability of A7r5 cells. After treatment with PTF for 24 (A), 48 (B) and 72h (C), respectively, the viability of A7r5 cells was analyzed by CCK-8 method. *P<0.05, **P<0.01 vs. the control (0.0mg/ml PTF). n=5.

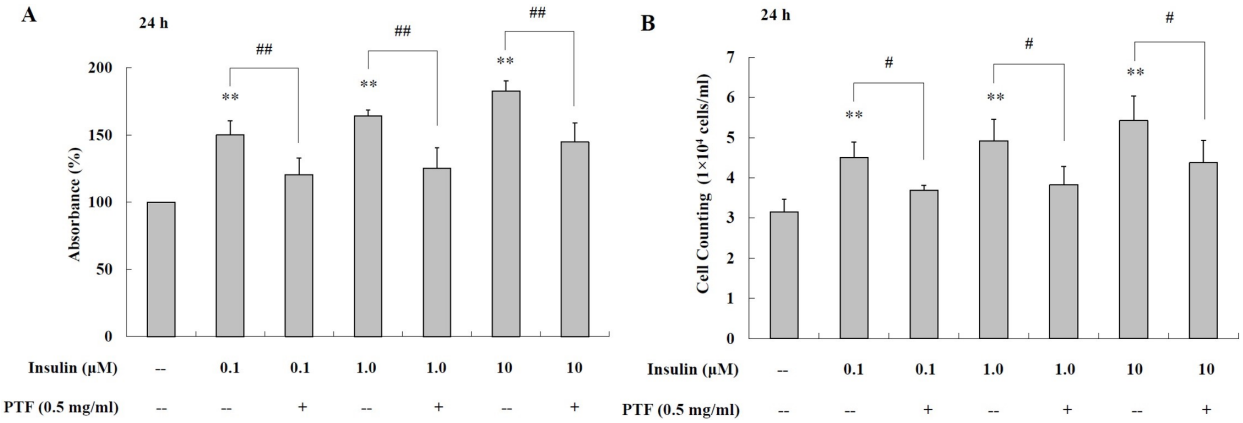

Fig. 4: Effects of PTF on different dose insulin-induced proliferation of A7r5 cells. After treatment with insulin (0.1~10μM) or/and PTF (0.5mg/ml) for 24h, the absorbance (A) and cell number (B) of A7r5 cells were analyzed by CCK-8 method. **P<0.01 vs. the control (free insulin); $^{\#}$P<0.05, $^{\#\#}$P<0.01. n=3.

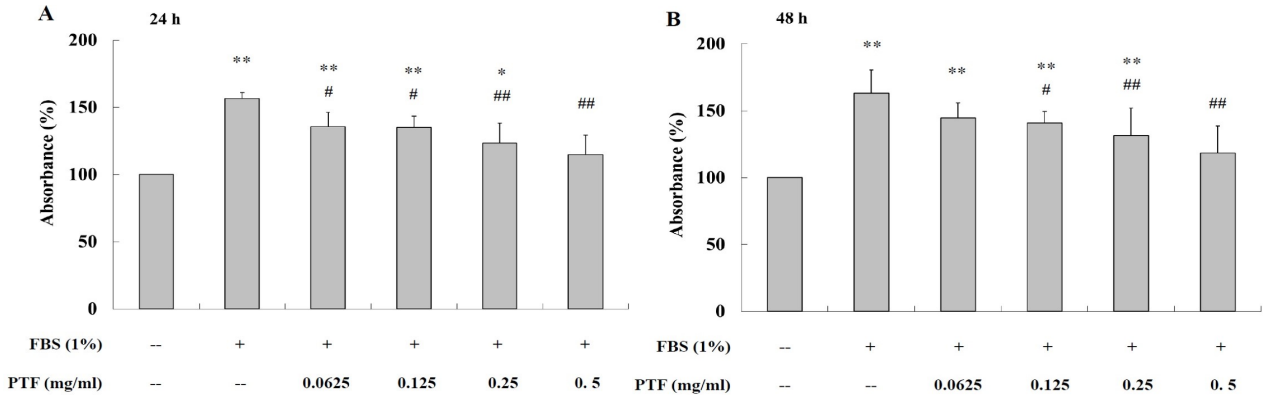

Fig. 5: Effects of PTF on 1%FBS-induced proliferation of A7r5 cells. After treatment with 1% FBS or/and PTF for 24 (A) and 48h (B), respectively, the proliferation of A7r5 cells was analyzed by CCK-8 method. *P<0.05, **P<0.01 vs. the control (free 1% FBS); $^{\#}$P<0.05, $^{\#\#}$P<0.01 vs. 1% FBS. n=3-4.

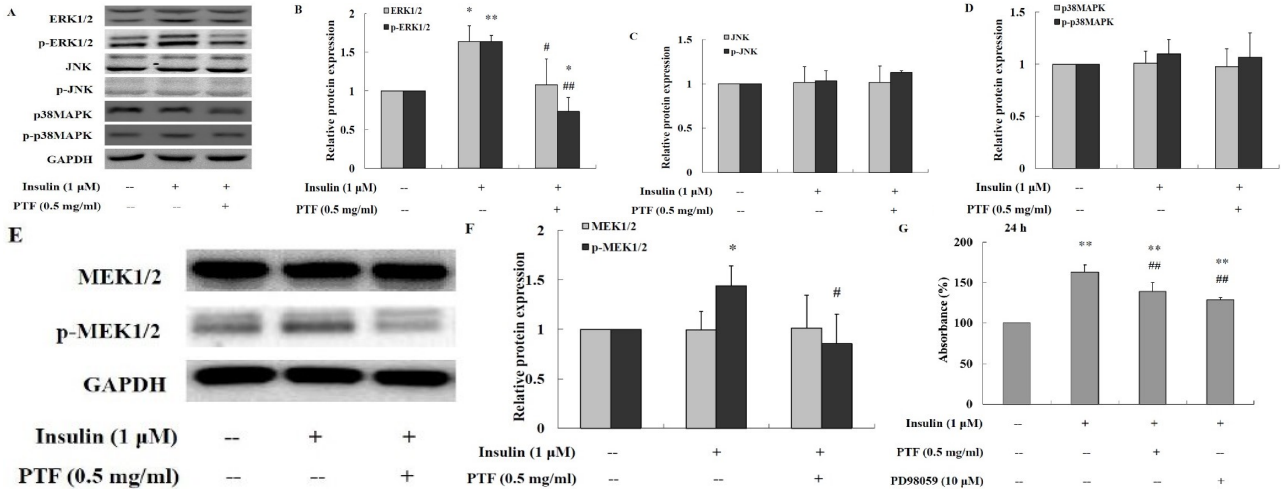

Fig. 6: Effects of PTF on the MAPK signaling pathway in A7r5 cells. (A, E) After treatment with insulin or/and PTF for 24 h, A7r5 cells were collected and lysed, the protein expression of ERK1/2, p-ERK1/2, JNK, p-JNK, p38MAPK, p-p38MAPK (A) as well as MEK1/2 and p-MEK1/2 (E) was checked by western blotting. (B) Densitometric analysis of ERK1/2 and p-ERK1/2. (C) Densitometric analysis of JNK and p-JNK. (D) Densitometric analysis of p38MAPK and p-p38MAPK. (F) Densitometric analysis of MEK1/2 and p-MEK1/2. (G) After treatment with insulin or/and PTF, PD98059 for 24 h, the proliferation of A7r5 cells was analyzed by CCK-8 method. *P < 0.05, **P < 0.01 vs. the control (free insulin); $^{\#}$P < 0.05, $^{\#\#}$P < 0.01 vs. 1 μM insulin.

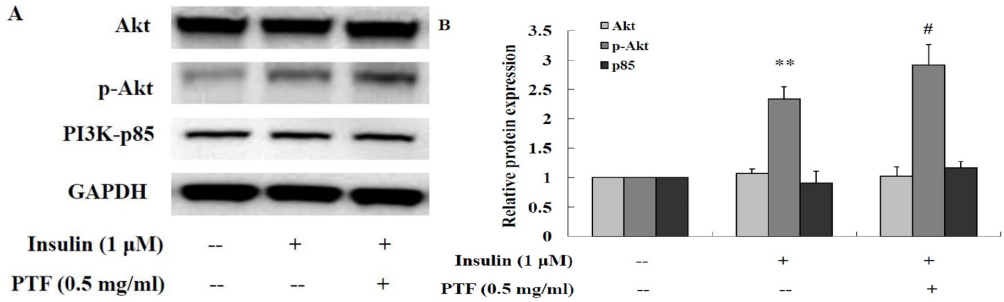

Fig. 7: PTF increases insulin-induced phosphorylation of Akt. After treatment with insulin or/and PTF for 24h, A7r5 cells were collected and lysed, the protein expression of Akt, p-Akt and PI3K-p85 (A) were analyzed using western blotting. (B) Densitometric analysis of Akt, p-Akt and PI3K-p85. **P<0.01 vs. the control (free insulin); $^{\#}$P<0.05 vs. 1μM insulin.

insulin sensitivity (Lou *et al.*, 2008). Indeed, ethanolic extracts of *Pollen typhae* exerts anti-inflammatory activity in lipopolysaccharide-induced macrophage (Chen *et al.*, 2021).

CONCLUSION

PTF suppresses insulin-promoted proliferation of A7r5 cells through at least in part, the MEK1/2-ERK1/2 cascades, which suggest new insight into the potential uses of PTF for treatment of diabetic atherosclerosis.

ACKNOWLEDGEMENT

This work was supported by the National Natural Science Foundation of China (81460711, 81760852), the Guangxi Natural Science Foundation (2014GXNSFAA118264), the Cultivation Program of 1000 Young and Middle-aged Backbone Teachers in Higher Education of Guangxi (Gui Teacher Education [2019]81), the Gui Style Xinglin Top Talent Funding Project of Guangxi University of Chinese Medicine (2022C012) and the Guangxi Collabrative Innovation Center for Scientific Achievements Transformation and Application on Traditional Chinese Medicine (05020058).

REFERENCES

Alcocer F, Whitley D, Salazar-Gonzalez JF, Jordan WD, Sellers MT, Eckhoff DE, Suzuki K, Macrae C and Bland KI (2002). Quercetin inhibits human vascular smooth muscle cell proliferation and migration. *Surgery*, **131**(2): 198-204.

Chen MT, Huang RL, Ou LJ, Chen YN, Men L, Chang X, Wang L, Yang YZ and Zhang Z (2019). Pollen typhae total flavone inhibits endoplasmic reticulum stress-induced apoptosis in human aortic-vascular smooth muscle cells through down-regulating PERK-eIF2α-ATF4-CHOP pathway. *Chin. J. Integr. Med.*, **25**(8): 604-612.

Chen P, Cao Y, Bao B, Zhang L and Ding A (2017). Antioxidant capacity of Typha angustifolia extracts and two active flavonoids. *Pharm. Biol.*, **55**(1): 1283-1288.

Chen S, Ding Y, Tao W, Zhang W, Liang T and Liu C (2012). Naringenin inhibits TNF-α induced VSMC proliferation and migration via induction of HO-1. *Food Chem. Toxicol.*, **50**(9): 3025-3031.

Chen T, Chen C, Huang Y, Baskaran R, Tsai JJP and Hu R (2021). Ethanolic extract of Puhuang (Pollen Typhae) modulates lipopolysaccharide-induced inflammatory response through inducible nitric oxide synthase/ cyclooxygenase-2 signaling in RAW 264.7 macrophage. *J. Tradit. Chin. Med.*, **41**(6): 836-844.

Chiu KM, Lin TY, Lee MY, Lu CW, Wang MJ and Wang SJ (2021). Typhaneoside suppresses glutamate release through inhibition of voltage-dependent calcium entry in rat cerebrocortical nNerve terminals. *Chem. Res. Toxicol.*, **34**(5): 1286-1295.

de Campos Zani SC, Son M, Bhullar KS, Chan CB and Wu J (2022). IRW (Isoleucine-Arginine-Tryptophan) improves glucose tolerance in high fat diet fed C57BL/6 mice via activation of insulin signaling and AMPK pathways in skeletal muscle. *Biomedicines,* **10**(6): 1235.

Duan H, Feng X and Huang X (2021). Effects of insulin on the proliferation and global gene expression profile of A7r5 cells. *Mol. Biol. Rep.*, **48**(2): 1205-1215.

Feng XT, Chen Q, Xie Z, Liang X, Jiang ZH, Zhao W and Leng J (2014). Pollen typhae total flavone improves insulin resistance in high fat diet and low dose streptozotocin-induced type 2 diabetic rats. *Biosci. Biotechnol. Biochem.*, **78**(10): 1738-1742.

Feng XT, Duan HM and Li SL (2017). Protective role of *Pollen typhae* total flavone against the palmitic acid-induced impairment of glucose-stimulated insulin secretion involving GPR40 signaling in INS-1 cells. Int. *J. Mol. Med.*, **40**(3): 922-930.

Feng XT, Wang TZ, Chen Y, Liu JB, Liu Y and Wang WJ (2012). Pollen typhae total flavone improves insulin-induced glucose uptake through the β-arrestin-2-mediated signaling in C2C12 myotubes. *Int. J. Mol. Med.*, **30**(4): 914-922.

Feng XT, Zhai LN, Wang CL, Zhao W, Chen Q and Huang XQ (2015). Effects of *Pollen typhae* total flavone on β-arrestin-2/Src/Akt signaling in adipose tissues of type 2 diabetic rats. *Afr. J. Tradit. Complement. Altern. Med*., **12**(5): 74-78.

Fu J, Yu MG, Li Q, Park K and King GL (2021). Insulin's actions on vascular tissues: Physiological effects and pathophysiological contributions to vascular complications of diabetes. *Mol. Metab.*, **52**: 101236.

Gao M, Ge Z, Deng R, Bao B, Yao W, Cao Y, Shan M, Cheng F, Yan H, Chen P and Zhang L (2021). Evaluation of VEGF mediated pro-angiogenic and hemostatic effects and chemical marker investigation for Typhae Pollen and its processed product. *J. Ethnopharmacol.*, **268**: 113591.

Hayfron-Benjamin CF, Amoah AGB, Maitland-van der Zee AH, Moll van Charante EP, Galenkamp H, van den Born BJ and Agyemang C (2021). Associations between macrovascular and renal microvascular dysfunction in type 2 diabetes and non-diabetes: The HELIUS study. *Microvasc. Res.*, **136**: 104162.

He W, Wang Y, Yang R, Ma H, Qin X, Yan M, Rong Y, Xie Y, Li L, Si J, Li X and Ma K (2022). Molecular mechanism of naringenin against high-glucose-induced vascular smooth muscle cells proliferation and migration based on network pharmacology and transcriptomic analyses. *Front. Pharmacol.*, **13**: 862709.

Huang S, Zhu X, Huang W, He Y, Pang L, Lan X, Shui X, Chen Y, Chen C and Lei W (2017). Quercetin inhibits pulmonary arterial endothelial cell transdifferentiation possibly by Akt and Erk1/2 pathways. *Biomed. Res. Int.*, **2017**: 6147294.

Kim SG, Sung JY, Kim JR and Choi HC (2020). Quercetin-induced apoptosis ameliorates vascular smooth muscle cell senescence through AMP-activated protein kinase signaling pathway. *Korean J. Physiol. Pharmacol.*, **24**(1): 69-79.

Lei X, Zhou Y, Ren C, Chen X, Shang R, He J and Dou J (2018). Typhae pollen polysaccharides ameliorate diabetic retinal injury in a streptozotocin-induced diabetic rat model. *J. Ethnopharmacol.*, **224**: 169-176.

Li F, Xia K, Sheikh SA, Cheng J, Li C and Yang T (2015). Involvement of RBP4 in hyperinsulinism-induced vascular smooth muscle cell proliferation. *Endocrine*, **48**(2): 472-482.

Li MF, Zhao CC, Li TT, Tu YF, Lun JX, Zhang R, Chen MY, Bao YQ, Li LX and Jia WP (2016). The coexistence of carotid and lower extremity atherosclerosis further increases cardio-cerebrovascular risk in type 2 diabetes. *Cardiovasc. Diabetol.*, **15**: 43.

Li W, Yu Y, Cheng H, Liu S, Gong T, Ma J and Tang Q (2022). Quercetin inhibits KBM7R cell proliferation through wnt/*β*-catenin signaling. *Evid. Based Complement. Alternat. Med.*, **2022**: 1378976.

Liu Q, Xiang P, Chen M, Luo Y, Zhao Y, Zhu J, Jing W and Yu H (2021). Nano-sized hydroxyapatite induces apoptosis and osteogenic differentiation of vascular smooth muscle cells via JNK/c-JUN pathway. *Int. J. Nanomedicine*, **16**: 3633-3648.

Lou SY, Liu Y, Chen WH, Ying J, He YM and Wang WJ (2008). Pollen typhae total flavones inhibit expression of interleukin-6 in C2C12 skeletal muscle cells cultured with palmitate. *Zhong Xi Yi Jie He Xue Bao*, **6**(5): 488-492.

Nhiem NX, Kiem PV, Minh CV, Lee JJ, Ku JH, Myung CS and Kim YH (2010). A potential inhibitor of rat aortic vascular smooth muscle cell proliferation from the Pollen of Typha angustata. *Arch. Pharm. Res.*, **33**(12): 1937-1942.

Peng Y, Cai P, Zou SF, Jia M, Zhong WT, Wang Y and Wang XK (2021). High dose insulin promotes the proliferation of vascular smooth muscle cells via AP-1/SM-α pathway. *J. Biol. Regul. Homeost. Agents*, **35**(3): 1029-1040.

Qiang D, Ci C, Liu W, Wang J, He C, Ji B and Shao X (2021). Inhibitory effect of kaempferol on mouse melanoma cell line B16 *In Vivo* and *In Vitro*. *Postepy Dermatol. Alergol.*, **3**8(3): 498-504.

Ruiz-Torres A1, Melón J and Muñoz FJ (1998). Insulin stimulates collagen synthesis in vascular smooth muscle cells from elderly patients. *Gerontology*, **44**(3): 144-148.

Safi A, Heidarian E and Ahmadi R (2021). Quercetin synergistically enhances the anticancer efficacy of docetaxel through induction of apoptosis and modulation of PI3K/AKT, MAPK/ERK and JAK/STAT3 signaling pathways in MDA-MB-231 breast cancer cell line. *Int. J. Mol. Cell. Med.*, **10**(1): 11-22.

Shi J, Wang A, Sen S, Wang Y, Kim HJ, Mitts TF and Hinek A (2012). Insulin induces production of new elastin in cultures of human aortic smooth muscle cells. *Am. J. Pathol.*, **180**(2): 715-726.

Tang H, Zeng Q, Tang T, Wei Y and Pu P (2021). Kaempferide improves glycolipid metabolism disorder by activating PPARγ in high-fat-diet-fed mice. *Life Sci.*, **270**: 119133.

Taniguchi CM, Emanuelli B and Kahn CR (2006). Critical nodes in signalling pathways: insights into insulin action. *Nat. Rev. Mol. Cell Biol.*, **7**(2): 85-96.

Wang D, Zhang X, Li D, Hao W, Meng F, Wang B, Han J and Zheng Q (2017). Kaempferide protects against myocardial ischemia/reperfusion injury through activation of the PI3K/Akt/GSK-3β pathway. *Mediators Inflamm.*, **2017**: 5278218.

Wang Y, Zhang Y, Gao X, Qian J, Yang J, Sun W, Wang H and Yang Y (2021). Resistin-like molecule beta augments phenotypic modulation of human aortic smooth muscle cell triggered by high glucose. *Endocr. J.*, **68**(4): 461-468.

Zeadin MG, Petlura CI and Werstuck, GH (2013). Molecular mechanisms linking diabetes to the accelerated development of atherosclerosis. *Can. J. Diabetes*, **37**(5): 345-350.

Zeng G., Wu Z, Cao W, Wang Y, Deng X and Zhou Y (2020). Identification of anti-nociceptive constituents from the pollen of Typha angustifolia L. using effect-directed fractionation. *Nat. Prod. Res.*, **34**(7): 1041-1045.

Zhang BF, Wu ZH, Deng J, Jin HJ, Chen WB, Zhang S, Liu XJ, Wang WT and Zheng XT (2021). M^6A methylation-mediated elevation of SM22α inhibits the proliferation and migration of vascular smooth muscle cells and ameliorates intimal hyperplasia in type 2 diabetes mellitus. *Biol. Chem.*, **403**(3): 317-329.

Zhang X, Yang K, Zhang H, Dong W, Peng W and Zhao Y (2020). Effect of typhaneoside on ventricular remodeling and regulation of PI3K/Akt/mTOR pathway. *Herz*, **45**(Suppl 1): 113-122.

Zhao J, Zhang CY, Xu DM, Huang GQ, Xu YL, Wang ZY, Fang SD, Chen Y and Gu YL (1990). The antiatherogenic effects of components isolated from pollen typhae. *Thromb. Res.*, **57**(6): 957-966.

Zhu HY, Huang ZX, Chen GQ, Sheng F and Zheng YS (2019). Typhaneoside prevents acute myeloid leukemia (AML) through suppressing proliferation and inducing ferroptosis associated with autophagy. *Biochem. Biophys. Res. Commun.*, **516**(4): 1265-1271.