

Paeoniflorin inhibits PDGF-BB-induced human airway smooth muscle cell growth and migration

HONG ZHOU¹, QI WU², LUQING WEI³ and SHOUCHUN PENG³

¹Graduate School of Tianjin Medical University, Tianjin 300070; ²Department of Respiration, Tianjin Medical University General Hospital, Tianjin 300052; ³Department of Respiratory and Critical Care Medicine, The Affiliated Hospital of Logistics University of Chinese People's Armed Police Force, Tianjin 300162, P.R. China

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Abstract. Abnormal proliferation and migration of airway smooth muscle cells (ASMCs) is important in the progression of asthma. Paeoniflorin (PF), one of the major active ingredients of *Paeonia lactiflora*, has been reported to exhibit anti-asthmatic effects. However, the effects of PF in the regulation of platelet-derived growth factor (PDGF)-BB-induced ASMC proliferation and migration remain unknown. The present study was designed to investigate the effects of PF on human ASMCs and the underlying mechanism. The results demonstrated that PF treatment significantly reduced the numbers of live ASMC cells and their PDGF-BB-induced migration. PF treatment also suppressed PDGF-BB-induced α -smooth muscle actin expression in ASMCs. Furthermore, pretreatment with PF reduced PDGF-BB-induced phosphorylation of phosphoinositide 3-kinase (PI3K) and AKT serine/threonine kinase 1 (Akt) in ASMCs. In conclusion, the present study demonstrated for the first time that PF inhibited ASMC growth and migration induced by PDGF-BB, and that this effect may be partly due to inhibition of the PI3K/Akt signaling pathway. The results provide novel information regarding the role of PF as a potential therapeutic agent for the treatment of asthma.

Introduction

Asthma is a chronic inflammatory disease of the lower airway, and its prevalence continues to increase in the past 20 years (1). Asthma is characterized by airflow obstruction, airway inflammation and airway remodeling (2). Despite many studies focusing on the pathogenesis of asthma, the underlying mechanisms remain unclear. Airway smooth muscle is a major

component of the remodeled airway in patients with asthma (3). Previous studies demonstrated that abnormal proliferation and migration of airway smooth muscle cells (ASMCs) is important in the pathogenesis of asthma (4,5). In addition, platelet-derived growth factor (PDGF)-BB has been reported to induce ASMC proliferation and migration (6,7), and elevated PDGF-BB expression is correlated with the severity of the disease (8). Therefore, inhibiting PDGF-BB-induced ASMC proliferation and migration may provide a novel therapeutic method for asthma.

Paeoniflorin (PF) is one of the major active ingredients of *Paeonia lactiflora*. There is substantial evidence that PF exhibits anti-inflammatory, antitumor, immunoregulatory, and nerve protective effects (9-12). For example, PF treatment significantly suppresses proliferation and invasion in human breast cancer cells (13). Recently, Sun *et al* (14) reported that PF oral administration in asthmatic mice markedly reduced airway hyperresponsiveness to aerosolized methacholine and decreased interleukin (IL)-5, IL-13, IL-17 and eotaxin levels in bronchoalveolar lavage fluid. However, the effects of PF on PDGF-BB-induced ASMC proliferation and migration remain unknown. The present study was designed to investigate the effects of PF treatment on human ASMCs and the underlying mechanism.

Materials and methods

Cell culture. The human ASMC line was purchased from the American Type Culture Collection (ATCC; Manassas, VA, USA) and cultured in DMEM (Invitrogen; Thermo Fisher Scientific, Inc., Waltham, MA, USA) medium containing 10% fetal bovine serum (FBS; Hyclone; GE Healthcare Life Sciences, Logan, UT, USA), 100 U/ml penicillin and 100 mg/ml streptomycin (Sigma-Aldrich; Merck KGaA, Darmstadt, Germany) in a humidified atmosphere of 5% CO₂ at 37°C. ASMCs in passages 9-20 were used for the experiments presented in this study.

Cell viability assay. Cell viability was measured using an MTT assay. In brief, ASMCs at a density of 1x10⁴ cells/well were seeded in a 96-well plate and grown in DMEM medium containing 10% FBS for 24 h. ASMCs were pretreated with PF (10, 20 and 40 nM; Sigma-Aldrich; Merck KGaA) for 1 h prior to stimulation with PDGF-BB (10 ng/ml; Sigma-Aldrich;

Correspondence to: Professor Qi Wu, Department of Respiration, Tianjin Medical University General Hospital, 154 Anshan Road, Tianjin 300052, P.R. China
E-mail: resp_wuqi@163.com

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Merck KGaA) for 24, 48 or 72 h. Then, MTT (5 mg/ml; Sigma-Aldrich; Merck KGaA) was added to each well and incubated for 4 h at 37°C in 5% CO₂. Subsequently, the supernatants were removed, and 150 µl of dimethyl sulfoxide was added to dissolve the formazan crystals. The absorbance at 450 nm was measured using a Spectra Max 190 microplate reader (Molecular Devices, LLC, Sunnyvale, CA, USA).

Cell migration assay. Cell migration was examined using transwell chambers. In brief, following treatment as described above, ASMCs at a density of 5x10⁴ cells/well suspended in 0.1% FBS medium were added into the upper chamber. DMEM medium (500 µl) containing 10% FBS, with or without PDGF-BB, was added into the lower chamber. Following incubation at 37°C for 24 h, cells migrated to the lower surface of the transwell membrane were stained with hematoxylin and eosin (Sigma-Aldrich; Merck KGaA), and the mean number of cells per four fields of view was counted under a light microscope. Migration was measured as the number of cells/field.

RNA extraction and reverse transcription-quantitative polymerase chain reaction (RT-qPCR). Total RNA was extracted from ASMCs using TRIzol reagent (Invitrogen; Thermo Fisher Scientific, Inc.), according to the manufacturer's instructions. cDNA was synthesized from the extracted RNA (3 µg) using the SuperScript First-Strand cDNA Synthesis SuperMix kit (Invitrogen; Thermo Fisher Scientific, Inc.). qPCR analysis was performed using a 7500 Real-Time PCR System (Applied Biosystems; Thermo Fisher Scientific, Inc.) with Fast Start Universal SYBR Green Master mix (Roche Diagnostics, Basel, Switzerland). The specific primers were: α-smooth muscle actin (α-SMA) sense, 5'-CTATTCCTTCGT GACTACT-3' and antisense, 5'-ATGCTGTTATAGGTGGTG GTT-3'; β-actin sense, 5'-TTAGTTGCGTTACACCCTTTC-3' and antisense, 5'-ACCTTCACCGTTCAGTTT-3'. The PCR cycling program was 95°C for 3 min, then 35 cycles of 94°C for 20 sec, 59°C for 30 sec and 72°C for 20 sec, and a final extension at 72°C for 5 min. β-actin was used as a normalization control and the results were analyzed by the method of 2^{-ΔΔCq} (15).

Western blot analysis. ASMCs were washed with PBS and lysed using radioimmunoprecipitation assay lysis buffer (Beyotime Institute of Biotechnology, Nantong, China). The cell lysate supernatants were harvested by centrifugation at 10,000 x g for 10 min at 4°C. The protein concentration was determined using a Bradford protein assay. Equal amounts of protein (30 µg) were separated by 10% SDS-PAGE and transferred onto polyvinylidene fluoride membranes (Sigma-Aldrich; Merck KGaA). Following blocking with 5% skim milk in Tris-buffered saline (TBS) with 0.1% Tween-20 at room temperature for 1 h, the membranes were incubated overnight at 4°C with primary antibodies targeting α-SMA (1:2,500; cat. no. sc-53142), phosphoinositide 3-kinase (PI3K, 1:2,000; cat. no. sc-365290), phosphorylated (p)-PI3K (1:2,500; sc-293115), AKT serine/threonine kinase 1 (Akt; (1:1,000; cat. no. sc-5298), p-Akt (1:1,000; cat. no. sc-52940) and GAPDH (1:3,000, cat. no. sc-59540; Santa Cruz Biotechnology, Inc., Dallas, TX, USA). Then, the membranes were washed with TBS containing 0.1% Tween for 10 min and incubated with

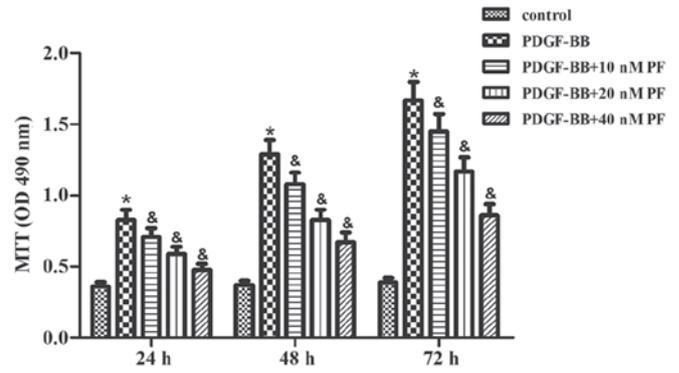


Figure 1. PF pretreatment suppresses PDGF-BB-induced ASMC growth. ASMCs were pretreated with PF (10, 20 or 40 nM) for 1 h prior to stimulation with PDGF-BB (10 ng/ml) for 24, 48 or 72 h. Untreated cells were used as control. Cell viability was measured with the MTT assay and reported as absorbance at 490 nm. Results are presented as mean + standard deviation of three independent experiments performed in triplicate. *P<0.05 vs. control group; &P<0.05 vs. PDGF-BB group. PF, paeoniflorin; PDGF-BB, platelet-derived growth factor-BB; ASMC, airway smooth muscle cells; OD, optical density.

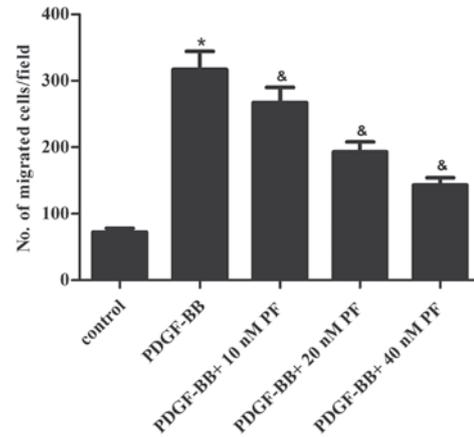


Figure 2. PF pretreatment suppresses PDGF-BB-induced ASMC migration. ASMCs were pretreated with PF (10, 20 or 40 nM) for 1 h prior to stimulation with PDGF-BB (10 ng/ml) for 24 h. Untreated cells were used as control. Cell migration was examined using Transwell migration chambers. Results are presented as mean ± standard deviation obtained from three independent experiments performed in triplicate. *P<0.05 vs. control group; &P<0.05 vs. PDGF-BB group. PF, paeoniflorin; PDGF-BB, platelet-derived growth factor-BB; ASMC, airway smooth muscle cells.

horseradish peroxidase-conjugated secondary antibodies (1:2,500, cat. no. sc-2005; Santa Cruz Biotechnology, Inc.) for 1 h at room temperature. Protein bands were evaluated by enhanced chemiluminescence (Thermo Fisher Scientific, Inc.). The optical densities of the bands were quantified using Gel-Pro Analyzer v4.0 (Media Cybernetics, Inc., Rockville, MD, USA).

Data analysis. The results were analyzed using SPSS software version 13.0 (SPSS, Inc., Chicago, IL, USA). All experiments were performed at least in triplicate and results are expressed as mean ± standard deviation. Statistical significance was analyzed with the one-way analysis of variance or the Student's two-tailed t-test followed by Tukey's post hoc test. P<0.05 was considered to indicate a statistically significant difference.

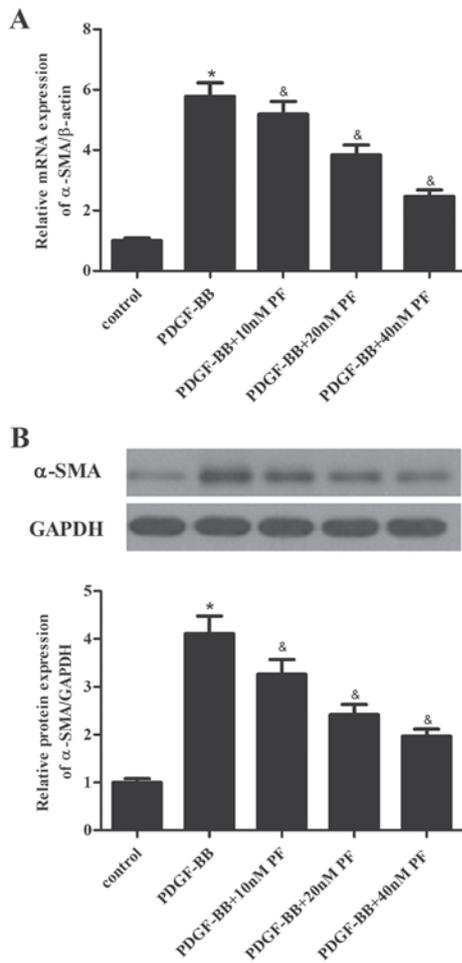


Figure 3. PF pretreatment suppresses PDGF-BB-induced α -SMA expression in ASMCs. ASMCs were pretreated with PF (10, 20 or 40 nM) for 1 h prior to stimulation with PDGF-BB (10 ng/ml) for 24 h. Untreated cells were used as control. (A) α -SMA mRNA expression levels were detected by reverse transcription-quantitative polymerase chain reaction. (B) α -SMA protein expression levels were detected by western blotting. Results are presented as mean \pm standard deviation of three independent experiments performed in triplicate. *P<0.05 vs. control group; [&]P<0.05 vs. PDGF-BB group. PF, paeoniflorin; PDGF-BB, platelet-derived growth factor-BB; α -SMA, α -smooth muscle actin; ASMC, airway smooth muscle cells.

Results

PF treatment suppresses PDGF-BB-induced ASMC growth. First, the effect of PF treatment on ASMC growth was examined using the MTT assay. As indicated in Fig. 1, PDGF-BB stimulation significantly induced growth in ASMCs over the course of 72 h, compared with the control group. However, the PDGF-BB-induced effect on ASMC growth was inhibited by PF pretreatment in a concentration-dependent manner (Fig. 1).

PF treatment suppresses PDGF-BB-induced ASMC migration. ASMC migration is hypothesized to be a key event in the pathogenesis of asthma (16). Thus, the effect of PF treatment on ASMC migration in response to PDGF-BB stimulation was examined. The results of transwell migration assays demonstrated that stimulation with PDGF-BB greatly promoted the migration of ASMCs, compared with the control group (Fig. 2). However, PF pretreatment partially reversed this effect in a dose-dependent manner (Fig. 2).

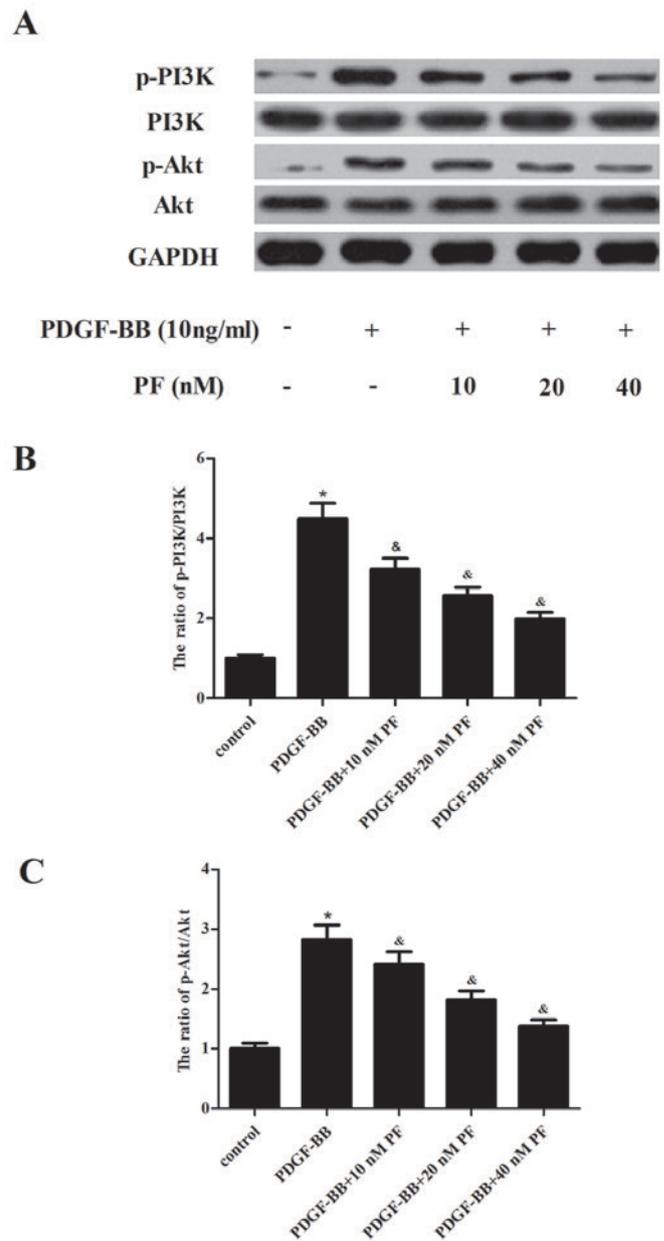


Figure 4. PF pretreatment inhibits PDGF-BB-induced phosphorylation of PI3K and Akt in ASMCs. ASMCs were pretreated with PF (10, 20 or 40 nM) for 1 h prior to stimulation with PDGF-BB (10 ng/ml) for 24 h. Untreated cells were used as control. The expression of p-PI3K, PI3K, p-Akt and Akt proteins was detected by western blotting. (A) Representative blots. (B) Quantification of the p-PI3K to PI3K ratio. (C) Quantification of the p-Akt to Akt ratio. Results are presented as the mean \pm standard deviation of three independent experiments performed in triplicate. *P<0.05 vs. control group; [&]P<0.05 vs. PDGF-BB group. PF, paeoniflorin; PDGF-BB, platelet-derived growth factor-BB; PI3K, phosphoinositide 3-kinase; Akt, AKT serine/threonine kinase 1; ASMC, airway smooth muscle cells; p, phosphorylated.

PF treatment suppresses PDGF-BB-induced α -SMA expression in ASMCs. α -SMA is a major constituent of the contractile apparatus of ASMCs, and its expression has been associated to the progression of asthma (17). Therefore, the effect of PF treatment on α -SMA expression in ASMCs was investigated. As demonstrated in Fig. 3A, PDGF-BB stimulation in ASMCs significantly increased the mRNA expression levels of α -SMA, compared with the control group. However, PF pretreatment

partially inhibited the PDGF-BB-induced α -SMA mRNA overexpression (Fig. 3A). Similarly, the results of western blot analysis demonstrated that PF pretreatment suppressed PDGF-BB-induced α -SMA protein overexpression in ASMCs, compared with cells treated with PDGF-BB alone (Fig. 3B).

PF treatment inhibits PDGF-BB-induced phosphorylation of PI3K and Akt in ASMCs. To explore the signaling mechanisms involved in the effects of PF on ASMC growth and migration, the phosphorylation status of PI3K and Akt was investigated by western blotting. As demonstrated in Fig. 4, PDGF-BB stimulation significantly activated PI3K and Akt phosphorylation in ASMCs compared with the control group. This activation was partially reversed by PF pretreatment, with phosphorylation levels for both PI3K and Akt significantly reduced compared with cells treated with PDGF-BB alone (Fig. 4).

Discussion

Abnormal proliferation and migration of ASMCs is involved in the progression of asthma (18). The present results demonstrated that PF treatment significantly inhibited PDGF-BB-induced ASMC growth and migration. PF pretreatment also suppressed PDGF-BB-induced α -SMA expression in ASMCs. Finally, PF pretreatment reduced PDGF-BB-induced phosphorylation of PI3K and Akt in ASMCs.

ASMCs obtained from asthmatic patients proliferate faster in culture than those obtained from non-asthmatic subjects (19). It has been reported that PF inhibits the proliferation of lung cancer A549 cells by blocking cell cycle progression in the G0/G1 phase (20). PF has also been demonstrated to be effective in the treatment of lung fibrosis in animal models (21). In the present study, PF treatment significantly inhibited PDGF-BB-induced growth of ASMCs, suggesting that PF may attenuate asthma progression via suppressing ASMC proliferation.

In addition, increased migration of ASMCs is thought to participate in the progression of asthma. ASMCs from patients with asthma exhibit more migratory capabilities compared with cells from normal subjects (22). A growing body of evidence indicates that PDGF-BB is a potent chemoattractant that induces ASMC migration (23-25). Thus, inhibition of ASMC migration represents a potentially important therapeutic strategy for the treatment of asthma. In the present study, PDGF-BB stimulation significantly induced ASMC migration, while PF pretreatment significantly inhibited this PDGF-BB-induced migration effect in ASMCs.

ASMC phenotypic switching (contractile to synthetic) is a critical mediator of airway remodeling in asthma. The phenotypic modulation of ASMCs contributes to the pathology of asthma by altering proliferation, secretion of inflammatory mediators and matrix deposition (26,27). It has been reported that increased levels of α -SMA facilitate ASMC proliferation and migration, which are important for asthma progression (28). *In vitro* PDGF-BB stimulation induces ASMC phenotypic switching (29,30). PF treatment has been demonstrated to decrease the contents of hydroxyproline, type I collagen and α -SMA in lung tissues of bleomycin-induced pulmonary fibrosis (31). In the present study, PF pretreatment suppressed PDGF-BB-induced α -SMA expression in

ASMCs, suggesting that PF may attenuate ASMC hyperplasia and hypertrophy.

PDGF-BB has been reported to stimulate proliferation and migration in ASMCs through PI3K signaling, which in turn activates Akt signaling (32). Inhibition of PI3K/Akt signaling significantly inhibits ASMC proliferation and migration (33,34). Furthermore, Zheng *et al* (35) reported that PF significantly inhibits the expression of PI3K, Akt, p-Akt and p-signal transducer and activator of transcription 3 in human gastric carcinoma MGC-803 cells, while a PI3K agonist (insulin-like growth factor 1) reverses the effect of PF on MGC-803 cell proliferation. In the present study, PDGF-BB treatment significantly activated PI3K and Akt phosphorylation in ASMCs. The present results are consistent with previous studies reporting that PI3K/Akt signaling mediates ASMC proliferation and migration induced by PDGF-BB. However, in the present study, PF pretreatment significantly suppressed PDGF-BB-induced phosphorylation of PI3K and Akt. These data suggest that PF inhibited PDGF-BB-induced human ASMC growth and migration through suppressing the activation of the PI3K/Akt signaling pathway.

In conclusion, the present study demonstrated for the first time that PF inhibited ASMC growth and migration induced by PDGF-BB. The inhibitory effect involved, at least partly, inhibition of the PI3K/Akt signaling pathway. Therefore, the present results provide evidence that PF may serve as a potential therapeutic agent for the treatment of asthma in the future.

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