**Original Article**

**Paeoniflorin inhibits PDGF-BB induced pulmonary arterial smooth muscle cells proliferation via p38 MAPK/JNK pathways and alleviate inflammation**

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**Abstract:** Objective: The objective of this study was to determine the effect of Paeoniflorin (PF), a monoterpene glycoside isolated from the roots of *Paeonia lactiflora* Pallas, on rat pulmonary arterial smooth muscle cells (PASMCs) pretreated with platelet-derived growth factor (PDGF)-BB. Methods: PASMCs were pretreated with PDGF-BB (60 ng/ml) or PF (20 µmol/L) under normal conditions. Cell viability was detected by Cell Counting Kit-8 (CCK-8) assay. Western Blot was used to measure the expression of p38 MAPK, ERK1/2, JNK, phospho-p38 MAPK (p-p38 MAPK), phospho-ERK1/2 (p-ERK1/2), and phospho-JNK (p-JNK). The cell cycle was analyzed by flow cytometry. Real-time PCR and enzyme-linked immunosorbent assay were used to measure the expression of pro-inflammatory cytokines. Results: CCK-8 analysis indicated that PDGF-BB induced the proliferation of PASMCs, and this effect was significantly inhibited by PF treatment (PF treatment = 0.63 ± 0.02, PDGF-BB = 0.71 ± 0.01, n = 3, P < 0.01). Under PDGF-BB pretreatment conditions, PF significantly decreased the proportion of S-phase (PF treatment = 24.63 ± 0.13, PDGF-BB = 51.35 ± 0.40, n = 3, P < 0.01) increased the proportion of G0/G1 and G2/M, and inhibited the phosphorylations of p38 MAPK and JNK, but not ERK1/2. In addition, PF markedly down-regulated the expression of PDGF-BB-induced pro-inflammatory cytokines, interleukin (IL)-1β, IL-6, tumor necrosis factor-α, and transforming growth factor-β1, in PASMCs. Conclusions: This study demonstrated that PF inhibited the proliferation of PASMCs induced by PDGF-BB. The underlying mechanism is in part mediated by the down-regulation of the activation of p38 MAPK/JNK pathways. Moreover, PF alleviated inflammation.

**Keywords:** Paeoniflorin, smooth muscle cells, mitogen-activated protein kinase, cytokine

**Introduction**

Pulmonary hypertension (PH) is a fatal disease and a common complication of chronic airway diseases [1]. Pulmonary artery vascular remodeling plays a crucial role in PH, and is related to pulmonary arterial smooth muscle cell (PASMCs) proliferation, migration, apoptosis and differentiation [2]. During this process, many growth factors and cytokines participate in cell proliferation, abnormal inflammation and vascular remodeling [3]. Platelet-derived growth factor (PDGF)-BB, serotonin, histamine and endothelin-1 have been reported to be involved in vascular remodeling [4]. PDGF-BB is a potent mitogen that participates in cell proliferation, survival and migration. There is growing evidence using numerous experimental models and in human patients that PDGF-BB is correlated with PH [5, 6].

Mitogen-activated protein kinases (MAPKs), including extracellular signal regulated kinase (ERK), c-Jun N-terminal kinases (JNK), and p38 MAPK, play an important role in the progression of cell proliferation, survival, or apoptosis [7]. Previous studies have reported that MAPKs are involved in vascular remodeling [8, 9]. PDGF-BB also induces PASMC proliferation and migration via the JNK, ERK1/2 or p38 MAPK signaling pathways [8, 10].

Paeoniflorin (PF), a monoterpene glycoside isolated from the roots of *Paeonia lactiflora* Pallas, which has many pharmacological effects including the inhibition of PASMC proliferation [11],
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Table 1. Primer sequences

<table>
<thead>
<tr>
<th>Target</th>
<th>Primer type</th>
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<td>Reverse</td>
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<tr>
<td>IL-6</td>
<td>Forward</td>
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<tr>
<td></td>
<td>Reverse</td>
<td>CCTCTTTGCTGCTTACACAT</td>
<td></td>
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<tr>
<td>TNF-α</td>
<td>Forward</td>
<td>GCTGCACCTTGGAGTGATC</td>
<td>136</td>
</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GTTGCTGACACATGCTGAGTA</td>
<td></td>
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<tr>
<td>TGF-β1</td>
<td>Forward</td>
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<td></td>
<td>Reverse</td>
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<td></td>
</tr>
<tr>
<td>β-Actin</td>
<td>Forward</td>
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</tr>
<tr>
<td></td>
<td>Reverse</td>
<td>GAGCCGCCATGCCAC</td>
<td></td>
</tr>
</tbody>
</table>

Figure 1. Cell viability of PASMCs was detected by CCK-8. PASMCs were pretreated with PDGF-BB (60 ng/ml) or PF (20 µmol/L) for 24 hrs. *P < 0.05 (compared with the control group); #P < 0.05 (compared with the PDGF group), n = 3.

anti-inflammatory actions [12, 13], and the activation of adenosine receptors [11, 14, 15]. We previously reported that PF inhibited the proliferation and promoted the apoptosis of PASMCs under hypoxic conditions and up-regulated the A2b adenosine receptor [11, 16]. Nizamutdinova et al. reported that PF inhibited tumor necrosis factor-α (TNF-α) induced NF-κB activation through the ERK1/2 and p38 MAPK, but not JNK, signaling pathways [17]. However, there is no currently available information on the role of PF in PDGF-induced PASMC responses.

In this study, we investigated the inhibitory role of PF on PDGF-BB-induced PASMC proliferation. We found that PF mediated its inhibitory effects via the p38 MAPK/JNK pathways, but not the ERK1/2 pathway, and alleviated the production of inflammatory mediators including interleukin-1β (IL-1β), IL-6, TNF-α, and transforming growth factor-β1 (TGF-β1).

Material and methods

Materials

PF was provided by the Shanghai Winherb Medical Technology Co., Ltd. (Shanghai, China). HPLC was used to validate the purity of PF, which was ≥ 98%. The concentration of PF was prepared as previously described [11]. PDGF-BB was obtained from R&D Systems (Minneapolis, MN, USA). Dulbecco’s Modified Eagle Medium (DMEM, high glucose), fetal bovine serum were purchased from Gibco BRL (Gaithersburg, MD, USA). Antibodies to p38 MAPK, p-p38 MAPK, ERK1/2, p-ERK1/2, JNK, and p-JNK were obtained from Cell Signaling Technology (Beverly, MA, USA). RevertAid First Strand cDNA Synthesis Kit was purchased from Fermentas (Waltham, MA, USA).

Cell culture

Rat PASMCs were obtained from male Sprague-Dawley adult rats (from the Laboratory Animal Center of Ningbo University, Ningbo, China) weighing 180-220 g. The detailed procedure was described previously [11].

Cell counting Kit-8 assay

The effect of PF on the proliferation of rat PASMCs was measured using a Cell Counting Kit-8 (CCK-8) assay. PASMCs were seeded into a 96-well plate at a density of 1×10^4 cells/well with DMEM. The PASMC cell cycles were synchronized by depriving them of serum for 24 hrs at 70-80% confluence. Then, the PASMCs were pretreated with PF (20 µmol/L) and PDGF-BB (60 ng/ml) in non-serum DMEM for 24 hrs. CCK-8 solution (10 µl/well) was added to each well. Absorbency was measured at 450 nm using a microplate reader (ELX800, BioTek Instrument, Winooski, VT, USA).

Flow cytometry

The serum-deprived cells were stimulated with PF in the presence or absence of PDGF-BB for 48 hrs. Then, cells were digested by trypsin, fixed in 75% ethanol and washed with PBS. The detailed procedure was previously described [11]. The cell cycle of stained cells was analyzed by flow cytometry (Becton, Dickinson and Company, NY, USA).
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Figure 2. Effects of PF on rat PASMC cell cycle. A. PASMC were grown under normal conditions. B. PASMCs treated with PDGF-BB (60 ng/ml). C. Cells pretreated with PF (20 µmol/L). D. Cells pretreated with PDGF-BB and PF. E. The proportion of cell cycle phases in PASMCs. *P < 0.05 (compared with the control group); #P < 0.05 (compared with the PDGF group), n = 3.
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Enzyme-linked immunosorbent assay

Cells were cultured for 24 hrs under PF and/or PDGF conditions. All the cells were collected and extracted by protein reagents on ice and centrifuged at 3600×g for 10 min to obtain the supernatant. This was then stored at -80°C until analysis. The concentrations of IL-1β, IL-6, TNF-α and TGF-β1 were detected by enzyme-linked immunosorbent assay (ELISA) according to the manufacturer’s protocol.

Western blot

The method used was previously described [11]. Briefly, protein from rat PASMCs was isolated after 24 hrs under PF or PDGF-BB conditions in ice-cold lysis buffer. Protein concentrations were measured using the Bradford protein assay. Then, protein samples were separated on 10% SDS-PAGE gels by electrophoresis and transferred to polyvinylidene fluoride membranes. The membranes were blocked by 5% skimmed milk in TBS-Tween 20 (0.1%, TBST), incubated with different primary antibodies at 4°C overnight, and then incubated with horseradish peroxidase-conjugated secondary antibodies. The membranes were incubated with ECL reagent for 1 min and exposed to X-ray film. The results were normalized to tubulin expression.

Quantitative real time polymerase chain reaction

As described previously [11], total RNA was extracted by Trizol reagent from rat PASMCs pretreated with or without PF. The isolated RNA was reverse-transcribed using a Prime Script reverse transcription reagent kit. The ABI 7300 system was used to analyze the expression levels. β-Actin was evaluated as a control gene and all the reactions were performed in triplicate. Data analyses were performed by the comparative Ct method using Applied Biosystems 7300 system SDS software. Specific primers used to detect IL-1β, IL-6, TNF-α, TGF-β1 and β-actin were shown in Table 1.

Statistical analysis

The results are shown as the mean ± SD. The results of statistical analyses performed by ANOVA were analysed by SPSS 16.0 software (IBM, USA) followed by the Newman-Student-Keuls test or Student t-test for differences between the two groups. Values of P < 0.05 were considered statistically significant.

Results

Inhibitory effect of PF on rat PASMC proliferation induced by PDGF-BB

Cells were pretreated with PDGF-BB (60 ng/ml) or PF (20 μmol/L) for 24 hrs. CCK-8 was performed to detect cell viability. As shown in

Figure 3. Effects of PF on the activation of MAPKs (A and B). PF did not affect ERK1/2, but inhibited p38 MAPK and JNK upon PDGF-BB stimulation in PASMCs.
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Figure 1, the absorbance of cells, which indicated the cell number, was significantly increased when incubated in PDGF-BB (0.71 ± 0.01) compared with the normal group (0.51 ± 0.00, P < 0.05, n = 3). However, the effect of PDGF-BB-induced PASMC proliferation was abolished by PF (0.63 ± 0.02, P < 0.05, n = 3).

Effects of PF and PDGF-BB on PASMC cell cycle

As shown in Figure 2, the proportion of S phase cell cycle was significantly increased in the pretreated PDGF-BB group compared to the normal group (51.35 ± 0.40% vs 42.27 ± 0.16%; P < 0.05, n = 3) and significantly decreased by PF (24.63 ± 0.13, compare to PDGF-BB group P < 0.01, n = 3) by flow cytometry. The proportion of G0/G1 (42.10% ± 0.33%) and G2/M (15.67% ± 0.18%) were significantly higher in the pretreated PDGF-BB group compared to the normal group (G0/G1 = 35.75 ± 0.39%) and (G2/M = 12.90 ± 0.24%), respectively (P < 0.01, n = 3).

PF inhibits PDGF-BB-induced p38 MAPK/JNK pathways

We detected the signal pathways involved in the process of PASMC activation induced by PDGF-BB. PF inhibited the phosphorylation of p38 MAPK and the JNK signaling pathway. However, PF did not influence the activation of the ERK1/2 signaling pathway (Figure 3).

PF modulates the release of PDGF-BB-induced inflammatory mediators

The mRNA and protein levels of IL-1β, IL-6, TNF-α, and TGF-β1 were measured by qPCR and
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ELISA at 24 hrs after PDGF-BB treatment. As shown in Figures 4 and 5, PDGF-BB enhanced pro-inflammatory cytokine production. However, these effects were almost completely reversed by PF treatment.

Discussion

It is well known that PDGF-BB induces the proliferation of smooth muscle cells. In the present study, we demonstrate that PF inhibits the proliferation of rat PASMCs induced by PDGF-BB. This is the first report, to the best of our knowledge, to show the involvement of inhibiting the phosphorylation of p38 MAPK/JNK signal pathways, but not ERK1/2, in the anti-proliferative activity of PF on rat PASMCs. We also demonstrated that PF suppressed the expression of pro-inflammatory cytokines including IL-1β, IL-6, TNF-α, and TGF-β1.

It was previously shown that PDGF-BB is involved in several cell-signaling cascades. PF suppressed activation of the NF-κB pathway [18] and the phosphorylation of JNK and p38 MAPK in human umbilical vein endothelial cells [19]. We previously reported that PF inhibited the proliferation and promoted the apoptosis of PASMCs under hypoxic conditions [11, 16]. However, the mechanisms involved in the effect of PF on PASMCs remains poorly understood. The present study indicated that PF inhibited the activation of p38 MAPK/JNK in PDGF-BB induced PASMCs, and that ERK1/2 was not involved. Cao et al. [12] performed cardiac ultrasonography to obtain a Doppler image from lipopolysaccharide (LPS)-challenged mice demonstrating that PF significantly increased the Left Ventricular Ejection Fraction (LVEF) and Fractional Shortening in PF-pretreated mice after LPS challenge. Thus, we can extend our understanding of pulmonary hypertension in human patients or animal models of hypertension.

Pro-inflammatory mediators, such as IL-1β, IL-6, TNF-α, and TGF-β1, play an important role in multiple organ failure and chronic diseases.
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They are especially involved in the vascular remodeling during pulmonary hypertension, and therefore it would be of benefit to inhibit their expression in PASMCs of pulmonary hypertension patients. High-dose PF is a potent anti-inflammatory agent of human dermal microvascular endothelial cells and inhibits the TNF-α-induced mRNA and protein expression of chemokines including CCL2, CCL5, CXCL8, CCL20, CXCL16 and CX3CL1 [20]. PF inhibited or partially regulated the production of TNF-α, IL-6 and IL-1β in LPS induced diseases [12, 21]. Therefore, the effect of PF on inflammatory mediators should be investigated further. We demonstrated that PF remarkably inhibited IL-1β, IL-6, TNF-α and TGF-β1 expression in rat PASMCs induced by PDGF-BB. Therefore, the production of pro-inflammatory cytokines can be suppressed by PF, which exerts an anti-inflammatory-like effect in PASMCs.

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Disclosure of conflict of interest

None.

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References


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