

Paeoniflorin Inhibits Pulmonary Artery Smooth Muscle Cells Proliferation via Upregulating A_{2B} Adenosine Receptor in Rat

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Abstract

Paeoniflorin (PF), which is the main active ingredient in the root of *Paeonia Radix*, has many pharmacological effects. Here, we investigated the effect of PF on rat pulmonary artery smooth muscle cells (PASMCs) under hypoxic conditions and explored the mechanisms of the effects. The anti-proliferative effect of PF increased in a dose dependent manner. At the highest dose (20 μmol/L), the anti-proliferative effect of PF peaked at 24 h after administration. However, the selective A_{2B} adenosine receptor (A_{2B}AR) antagonist MRS1754 abolished it. PF increased A_{2B}AR mRNA levels from 0.0763±0.0067 of β-actin mRNA levels (hypoxia group) to 0.1190±0.0139 (P<0.05) measured by Real Time Reverse Transcription-Polymerase Chain Reaction. A_{2B}AR protein expression measured by Western Blot was also increased. PF inhibited the proliferation of PASMCs by blocking cell cycle progression in the S phase. These data indicated that activation of A_{2B}AR might be involved in the anti-proliferative effect of PF on PASMCs under hypoxic conditions. The results suggested that a new mechanism of PF could be relevant to the management of clinical hypoxic pulmonary hypertension.

Citation: Qian G, Cao J, Chen C, Wang L, Huang X, et al. (2013) Paeoniflorin Inhibits Pulmonary Artery Smooth Muscle Cells Proliferation via Upregulating A_{2B} Adenosine Receptor in Rat. PLoS ONE 8(7): e69141. doi:10.1371/journal.pone.0069141

Editor: Chiara Bolego, University of Padua, Italy

Received: February 22, 2013; **Accepted:** June 12, 2013; **Published:** July 30, 2013

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Funding: This study was supported by National Natural Science Foundation of China (No. 81270110), <http://www.nsf.gov.cn/Portal0/default152.htm> Zhejiang Provincial Natural Science Foundation of China (No. Y2101208), <http://www.zjnsf.gov.cn/>. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Pulmonary arterial hypertension (PAH), defined as a mean pulmonary artery pressure (PAPm) ≥25 mmHg with a pulmonary capillary wedge pressure ≤15 mmHg measured by cardiac catheterization [1]. PAH contributes to the morbidity and mortality of patients with various diseases [2]. The pathogenesis of PAH is complex and poorly understood, but chronic hypoxia is suspected as a cause of the structural changes in pulmonary arteries which might be a factor in the pathogenesis of PAH. Recent research reported that pulmonary vascular remodeling plays a key role in pulmonary arterial hypertension, which is partly due to the proliferation of pulmonary artery smooth muscle cells (PASMCs).

Adenosine receptors, which are extracellular G protein-coupled receptors, namely, A₁, A_{2A}, A_{2B}, A₃, mediate adenosine actions [3]. As A_{2B} adenosine receptor (A_{2B}AR) have a lower affinity compared to other subtypes, they require micromolar concentrations of adenosine for stimulation [4]. Such high levels of extracellular adenosine are generated or released from cell under stress like hypoxia, ischemia, inflammation, and injury [5,6]. Adenosine signaling through A_{2B}AR has been shown to inhibit

smooth muscle cell proliferation [7,8], and prevent additional injury of cardiac tissues post-infarction [9].

Paeoniflorin (PF), the principal bioactive component of *Paeoniae Radix* [10], has been reported to have many pharmacological effects such as decreasing pulmonary artery pressure [11], relaxing vascular smooth muscle [12], analgesic [13], anti-inflammatory and anti-allergic [14], and cognition-enhancing effects [15] and the ability to activate A₁ and A_{2A} adenosine receptors [10,16,17]. Whether PF has an effect on A_{2B}AR in PASMCs was unknown. Accelerated proliferation of PASMCs plays a critical role in the progression of PAH. Therefore, finding new inhibitors of PASMC proliferation is an important strategy in the identification of new therapies for PAH. The goal of this study was to investigate the effects of PF on rat PASMCs under hypoxic conditions and their possible mechanisms.

Materials and Methods

Materials

PF (Figure 1) was purchased from Silida Technology Ltd. (Tianjing, China); with a purity of ≥98.5% as determined by HPLC. PF dissolved in 50 ml sterilized saline water to prepare for stock solution with 200 μmol/L; Dilution was obtained by adding

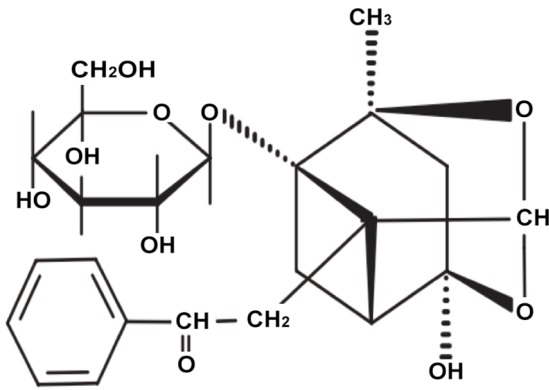


Figure 1. Chemical structure of paeoniflorin.
doi:10.1371/journal.pone.0069141.g001

saline, and the dose was used variedly according to different administrations. Fetal bovine serum (FBS), penicillin G, streptomycin, Dulbecco's Modified Eagle Medium (DMEM, high glucose) were obtained from Gibco BRL (Gaithersburg, MD, USA). DPCPX, MRS1754, SCH58261 and IB-MECA, respectively, the selective antagonist for A₁, A_{2A}, A_{2B}, A₃ adenosine receptor and Dimethylsulphoxide (DMSO), ribonuclease (RNase), propidium iodide (PI), collagenase type I were all obtained from Sigma Chemical (St Louis, MO, USA). Cell counting kit-8 (CCK-8) was obtained from Dojindo Laboratories (Kumamoto, Japan). Cell cycle staining kit was purchased from Multiscience Biotech Co., Ltd. (Shanghai, China). Trizol was obtained from Invitrogen (Carlsbad, CA, USA). TaqMan polymerase was purchased from Promega (Madison, Wis, USA). SYBR Green Real-Time PCR was obtained from Toyobo (Osaka, Japan). A_{2B}AR polyclone antibody was from Santa cruz Biotechnology, Inc (Santa cruz, CA, USA).

Cell culture

Male Sprague-Dawley adult rats (obtained from the laboratory animal center of Wenzhou Medical College, Wenzhou, Zhejiang, China) weighing 180–220 g were used throughout the study. All the animal procedures in the present study carried out in

accordance with the guidelines of Wenzhou Medical College and National Institutes of Health standards of animal care. Our study was approved by the Animal Ethics Committee of Wenzhou Medical College including permit number SCXK (Zhejiang 22–07168). The pulmonary artery from male Sprague-Dawley rats was removed under sterile conditions and washed in D-Hank's solution. The outer and inner membranes were removed under anatomy microscope. Minced arteries were digested by 0.2% collagenase type I, then incubated in 37°C for 1–2 h. The digested pulmonary arteries were centrifuged at 1000 rpm for 7 min and then suspended by DMEM containing 20% FBS. After being dispersed, they were placed in 60 mm culture dish to incubate in incubator. The medium was changed after 72 h. Cells were maintained in the medium until 80%–90% confluence and they were subcultured with trypsin (0.25%)–EDTA (0.02%). Cells were used at passages 4–10 for experiments. The change of cells morphology was not noted and cells were identified positive of α -smooth muscle actin by immunofluorescence. Cells were made quiescent by incubation in serum-free DMEM for 24 h for all experiments. Hypoxia cells were performed in a CO₂-N₂ incubator (Heraeus, Germany) at 5% O₂, 5% CO₂, and 90% N₂, 37°C. PASMCS were divided into 9 groups: (1) Normal (N), in which cells were cultured in serum-free DMEM under normoxia (21% O₂, 5% CO₂) for 24 h; (2) Normoxia + paeoniflorin (N+PF20), in which cells were cultured in serum-free DMEM with paeoniflorin (20 μ mol/L) for 24 h under normoxia conditions; (3) Hypoxia (H), in which cells were cultured in serum-free DMEM under hypoxia (5% O₂, 5% CO₂) for 24 h; (4) Hypoxia +0.02 μ mol/L paeoniflorin (H+PF0.02), in which cells were cultured in serum-free DMEM with paeoniflorin (0.02 μ mol/L) for 24 h under hypoxia; (5) Hypoxia +0.2 μ mol/L paeoniflorin (H+PF0.2); (6) Hypoxia +2 μ mol/L paeoniflorin (H+PF2); (7) Hypoxia +20 μ mol/L paeoniflorin (H+PF20); (8) Hypoxia + MRS1754 (H+MRS), in which cells were cultured in serum-free DMEM with MRS1754 (a selective antagonist for A_{2B} adenosine receptor, 20 nmol/L) for 24 h under hypoxia; (9) Hypoxia +20 μ mol/L paeoniflorin + MRS1754 (H+PF20+MRS), in which cells were cultured in serum-free DMEM with paeoniflorin (20 μ mol/L) and MRS1754 (20 nmol/L) for 24 h under hypoxia.

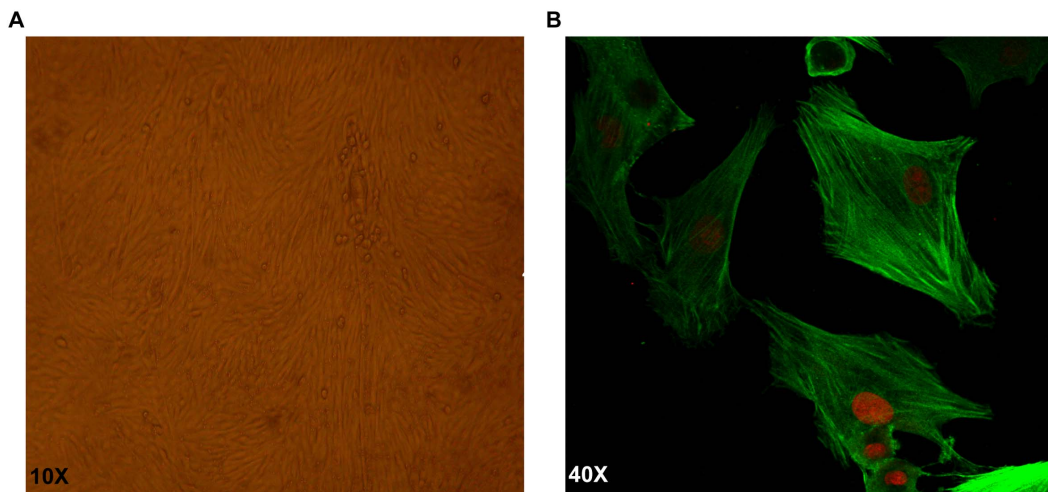


Figure 2. PASMCS morphology. A) Typical "hill and valley" appearance of PASMCS under phase contrast microscope. B) Immunofluorescent identification of α -smooth muscle actin was positive.
doi:10.1371/journal.pone.0069141.g002

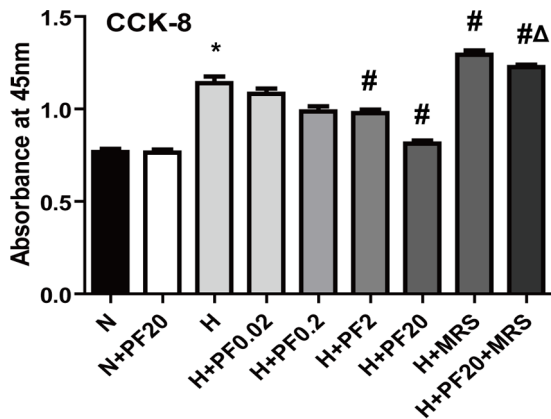


Figure 3. Cell viability was tested with CCK-8. * $P < 0.05$ vs. the N group; # $P < 0.05$ vs. the H group; Δ $P < 0.05$ vs. the H+PF20 group. Mean \pm SD, $n = 6$. doi:10.1371/journal.pone.0069141.g003

Cell proliferation assay

Cell anti-proliferation by PF was detected by the CCK-8 assay. Briefly, PSMCs cultured in 96-well plates (1×10^4 cells/well) in the complete medium (5% CO_2 , 37°C , 95% humidity) for 24 h and then put into hypoxia incubator as described above (6 wells in each group). CCK-8 solution (10 μl /well) was added to each well. After incubate for 1 h at 37°C , the absorbance of each well was determined using a microplate reader (ELX800, BioTek Instruments, Winooski, VT, USA).

Flow cytometry

To determine cell cycle distribution, 5×10^5 cells were plated in 60 mm dishes and treated without or with PF at concentrations (20 $\mu\text{mol/L}$) for 24 h. Then, the cells were digested by trypsinization, after fixed in 75% ethanol, the cells were washed with phosphate buffered saline (PBS), and aliquots of the cells were resuspended in 1 ml of PBS containing 1 mg/ml of RNase and 50 $\mu\text{g/ml}$ of propidium iodide (PI). After put in a dark place for

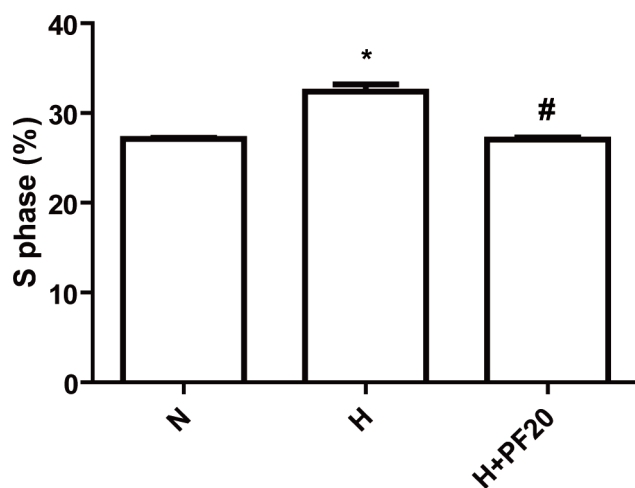


Figure 4. Paeoniflorin decreased the proportion of S phase in hypoxic PSMCs. PSMCs were incubated under normoxic (N), hypoxic (H) condition, or pretreated with PF (20 $\mu\text{mol/l}$) when under hypoxic condition. * $P < 0.05$ vs. the N group; # $P < 0.05$ vs. the H group; $n = 3$. doi:10.1371/journal.pone.0069141.g004

30 min at 25°C , the cell cycle was analyzed using flow cytometer (Becton, Dickinson and Company, NY, USA).

Quantitative Real Time Polymerase Chain Reaction

We used the Trizol extract total RNA from PSMCs. RNA concentration was quantified using absorbance at 260 nm and the ratio of the absorbance at 260 and 280 nm (A_{260}/A_{280}) was used to assess the purity of RNA. Reverse Transcription was performed using the Bio-Rad PCR system S1000 (Bio-Rad, Hercules, CA, USA) with 2 μl cDNA in a reaction volume of 8 μl containing 1 μl $10 \times$ PCR buffer and 0.8 μl primer mix. The final reverse transcription product was adjusted to 8 μl with RNase-free water. The specific primers for adenosine receptors and GAPDH: A_1 (forward: 5'GTGATTTGGGCTGTGAAGGT3'; reverse: 5'AGTGTGTGGAAGTAGGTCTGTGG3'), A_{2A} (forward: 5'CGGG-AACTCCACGAAGACC3'; reverse: 5'AGCAAAGAGCCCCGACGATG3'), A_{2B} (forward: 5'TCTTCCTCGCCTGCTTCGT3'; reverse: 5'GGAGTCAGTCCAATGCCAAA3'), A_3 (forward: 5'GAAAGCCAACAATACCACGAC3'; reverse: 5'AGTGCTAGGGAGACGATGAAAT3'), and GAPDH (forward: 5'GGAAA-GCTGTGGCGTGAT3'; reverse: 5'AAGGTGGAAGAATGGGAGTT3') were designed by Primer 5.0. PCR was performed with the 7500 Real-Time PCR instrument (Applied Biosystems, Carlsbad, CA, USA). All the reactions were performed in triplicate and normalized using GAPDH as control gene. All data of the Real-Time PCR was analyzed using the Applied Biosystems 7500 System SDS Software using the standard curve method. For data analyses, the $2^{-\Delta\Delta\text{CT}}$ values were determined.

Western Blot analysis

Western Blot was used to detect A_{2B} adenosine receptor. Briefly, PSMCs were harvested, pelleted, and resuspended in ice cold lysis buffer. The Bradford protein assay was used to measure the concentration of protein. SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred onto a polyvinylidene fluoride (PVDF) membrane (Millipore, Billerica, MA, USA). The free protein binding sites were blocked by incubating with PBS containing 5% skimmed milk at 4°C overnight. The blot was then washed five times before incubation with goat anti-rabbit IgG in the blocking solution for 1 h. Wash the membrane again as before. Then the protein bands for other antigen-antibody complex were detected by ECL plus detection system. After scanning the X-ray film, the optical density of the immunoblots was calculated with the Quantity one-4.6.2 software (Bio-Rad, Hercules, CA, USA). A membrane incubated with a HRP-conjugated polyclonal anti- β -actin served as the control.

Statistics

All data were shown as the mean \pm the standard derivation (SD). Statistical analysis of the results were counted by one-way ANOVA followed by a Student-Newman-Keuls test. A $P < 0.05$ was considered statistically significant.

Results

The morphology and identify of PSMCs

As shown in Figure 2A and Figure 2B, confluent PSMCs manifested typical "hill and valley" features under a phase-contrast microscope, and were positive for α -smooth muscle actin by Immunofluorescent staining.

Effect of hypoxia and PF on proliferation of PSMCs

The absorbance of PSMCs, which indicated the cell number, increased under hypoxic conditions when cells were cultured in

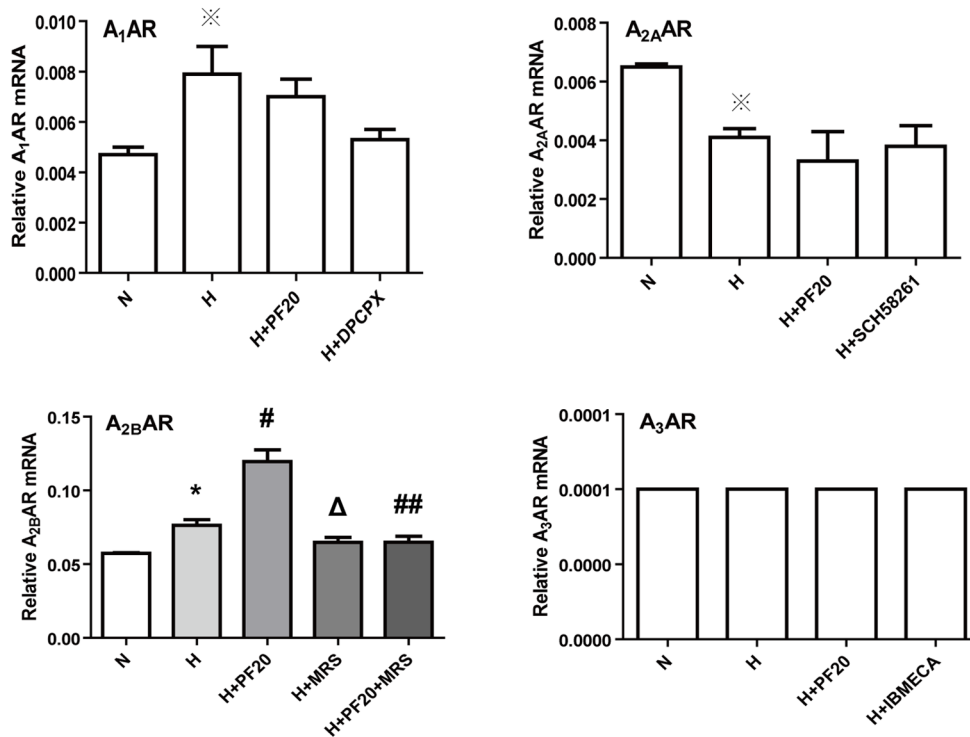


Figure 5. Effects of hypoxia and paeoniflorin on the expression of adenosine receptors. The PSMCs were incubated under normoxic (N), hypoxic (H) condition, or pretreated with PF (20 μ mol/l) when under hypoxic condition. \ast $P > 0.05$ vs. the N group; \ast $P < 0.05$ vs. the N group; $\#$ $P < 0.05$ vs. the H group; $\#\#$ $P < 0.05$ vs. the H+PF20 group; Δ $P < 0.05$ vs. the H group $n = 3$. doi:10.1371/journal.pone.0069141.g005

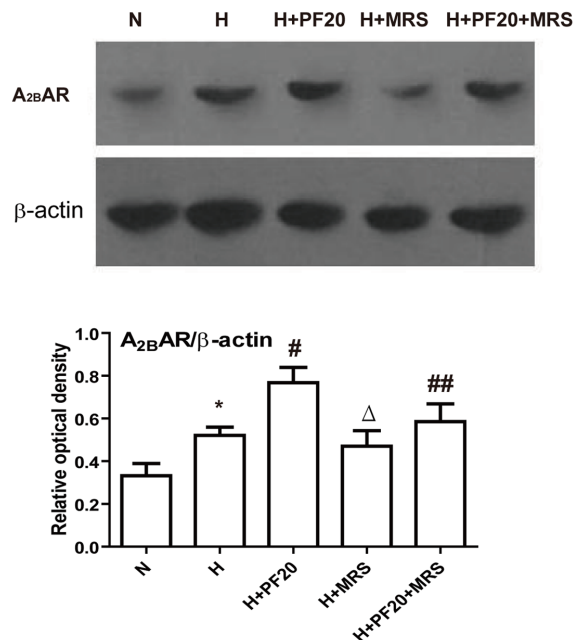


Figure 6. Paeoniflorin upregulate A_{2B}AR protein expression in hypoxic PSMCs. Treat PSMCs were assayed for A_{2B}AR protein. \ast $P < 0.05$ vs. the N group; $\#$ $P < 0.05$ vs. the H group; $\#\#$ $P < 0.05$ vs. the H+PF20 group; Δ $P < 0.05$ vs. the H group. $n = 3$. doi:10.1371/journal.pone.0069141.g006

complete medium with 10% FBS (Figure 3). PF suppressed the proliferation of PSMCs in a dose-dependent manner between 0.02 μ mol/L and 20 μ mol/L. At a PF concentration of 20 μ mol/L, the proliferation of PSMCs was significantly suppressed ($P < 0.05$ versus hypoxic group, $P > 0.05$ versus normoxic group, $n = 6$). The anti-proliferative effect of PF was abolished by the selective A_{2B}AR antagonist MRS1754 (20 nmol/L).

Cell cycle analysis by Flow Cytometry

The effects of PF on cell cycle progression of PSMCs are shown in Figure 4. Compared with the normoxic group, the proportion of cells in the S phase cells was significantly increased from $27.10 \pm 0.15\%$ to $32.37 \pm 0.80\%$ in the hypoxic population ($P < 0.05$). After treatment with 20 μ mol/L PF under hypoxic conditions, the proportion cells in the S phase decreased to $27.06 \pm 0.24\%$, indistinguishable from the normoxic group.

Effects of hypoxia and PF on the expression of adenosine receptors

Quantitative Real-time PCR was used to measure the mRNA levels of the different adenosine receptors in PSMCs. As shown in Figure 5C, A_{2B}AR had the highest expression levels among the four subtypes of adenosine receptor under normoxic conditions. A₁AR, A_{2A}AR and A₃AR were also detected. The expression of A₃AR was the lowest (Figure 5A, B, D). The expression levels of adenosine receptors ranked in the following order A_{2B}AR > A_{2A}AR > A₁AR > A₃AR. Under hypoxic conditions too, A_{2B}AR was the predominant adenosine receptor subtype expressed in PSMCs (Figure 5C). The mRNA levels of A_{2B}AR were increased from 0.0573 ± 0.0008 to 0.0763 ± 0.0067 ($P < 0.05$), but the levels of

A₁AR, A_{2A}AR or A₃AR showed little change. After pretreatment with PF under hypoxic conditions, the expression levels of A_{2B}AR increased significantly to 0.1190 ± 0.0139 ($P < 0.05$, versus to hypoxic control group). As shown in Figure 6, the expression of A_{2B}AR protein was detected by Western Blot. A_{2B}AR protein expression increased in the hypoxic control group compared to the normoxic control group. PF induced a further increase in the A_{2B}AR protein levels in hypoxic rat PSMCs. However, the effects of PF, which increased the expression of A_{2B}AR mRNA and protein under hypoxic conditions, were abolished by MRS1754 (Figure 5C, Figure 6).

Discussion and Conclusions

To our knowledge, this is the first report about the effects of PF and hypoxia on rat PSMCs, and a novel mechanism is proposed for the role of PF and A_{2B}AR in hypoxic pulmonary hypertension. PF caused inhibition of pulmonary artery smooth muscle cells growth by activating A_{2B}AR and inducing its expression.

Several reports demonstrated the presence of adenosine receptors in lung in different species. A₁AR, A_{2B}AR, and A₃AR were detected in rat airway smooth muscle cells [18]. It was reported that the levels of A_{2B}AR transcripts were the highest in human bronchial smooth muscle cells, A₁AR and A_{2A}AR transcripts were detected also, but A₃AR transcripts were below detection limit [19]. Similarly, our study also shows that among the four subtypes of adenosine receptors in rat PSMCs, the expression of A_{2B}AR was highest with lower levels of A₁AR and A_{2A}AR. However, A₃AR mRNA was also detected at a lower level. The differences of adenosine receptors between rat and human are not entirely surprising because it is possible that the effects of hypoxia on the expression of adenosine receptors are cell- and tissue-specific [20,21].

Hypoxia is common in a variety of disease states, like hypoxic pulmonary hypertension, tumor growth, and ischemia etc. Cell hypoxia is a potent stimulus for adenosine release. One study demonstrated that hypoxia downregulated high-affinity A_{2A}AR and upregulated low-affinity A_{2B}AR in Human Umbilical Vein Endothelial Cells [21]. In other reports A_{2B} adenosine receptors were upregulated under hypoxic conditions [22–24]. Here, we demonstrated that the expression of certain adenosine receptor subtypes in rat PSMCs changed greatly under hypoxic conditions. Hypoxia upregulated the expression of low-affinity

A_{2B}AR, but it did not change the expressions of A₁AR, A_{2A}AR, and A₃AR.

Earlier reports showed that A_{2B}AR is a tissue protector and that it inhibited Vascular Smooth Muscle Cell proliferation [8,25,26]. However, whether the activation of A_{2B}AR in PSMCs could inhibit PSMCs proliferation was unknown. The key finding of our study is that A_{2B}AR can inhibit the proliferation of PSMCs under hypoxic conditions.

PF is the main active ingredient in the root of *Paeonia Radix*, as Chinese herbal medicine. It has been reported to have many pharmacological effects. Many researchers focused on the effects of paeoniflorin on lung [11], brain [27], or plasma [28] etc. It was reported that PF could inhibit the proliferation in pig PSMCs [11], however, the mechanism of this paeoniflorin effect was not clear. Liu *et al* reported the activation of A₁AR might be involved in PF-induced neuroprotection in cerebral ischemia in rats, but this binding manner was different from that of classical A₁ adenosine receptor agonists [10]. PF increased erythropoietin mRNA levels by activating PI-3 kinase pathway through A₁ and A_{2A} adenosine receptors under hypoxic conditions [29]. Previously, it was unknown whether PF can upregulate A_{2B}AR under hypoxic conditions. Here we showed that pretreatment of PSMCs with under hypoxic conditions significantly upregulated the expression of A_{2B}AR, while it did not change the expression of A₁, A_{2A}, and A₃ adenosine receptors. To further investigate the possible mechanism underlying the effect of PF on A_{2B}AR, the PSMCs were pretreated with MRS1754, a selective A_{2B}AR antagonist which abolished PF-induced upregulation of A_{2B} adenosine receptors. To further clarify the detailed mechanism, more studies are required.

In conclusion, PF is effective in inhibiting the proliferation of PSMCs via upregulating the expression of A_{2B} adenosine receptors and by activating it. These findings might provide a new theoretical basis for the use of PF in the management of clinical hypoxic pulmonary hypertension.

Author Contributions

Conceived and designed the experiments: GQ LW J. Cao CC XH CD XC. Performed the experiments: GQJ. Cao CC XC FY J. Chu GL JY. Analyzed the data: GQJ. Cao CC XH. Contributed reagents/materials/analysis tools: GQ CC CD. Wrote the paper: GQJ. Cao. Guided the experiments and edited the manuscript: LW. Revised the manuscript: LW J. Cao CC.

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