Protective effect of baicalin against pulmonary arterial hypertension vascular remodeling through regulation of TNF-α signaling pathway

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Abstract

Pulmonary arterial hypertension (PAH) is a progressive cardiovascular disease with high mortality. However, there were no efficient medical drugs for PAH to enormously improve the survival and quality of life measures. The present study aimed to explore the protective effect of baicalin against experimental PAH in vivo and vitro. All the experimental rats received intraperitoneal injection of monocrotaline (MCT) to induce PAH model. Baicalin was given by intragastric administration from 2 days after MCT injection.Forty animals were randomly divided into four groups: Control, MCT, saline-, and baicalin-treated groups (n = 10 in each). Post-operation, hemodynamic data, and index of right ventricular hypertrophy (RVHI) were recorded to evaluate the inhibition of baicalin on MCT-induced PAH. Furthermore, pulmonary artery smooth muscle cells (PASMCs) model induced by tumor necrosis factor-α (TNF-α) was used to observe the inhibition of vascular cells proliferation in vitro. The results demonstrated that baicalin significantly attenuated MCT-induced right ventricular systolic pressure (RVSP), the index of right ventricular hypertrophy, and vascular wall thickness; inhibit inflammatory and cell proliferation induced by MCT or TNF-α, respectively. In addition, we found that baicalin might protect against experimental PAH via regulating the TNF-α/BMPR2 signaling pathway.

KEYWORDS
Baicalin, BMPR2, PAH, TNF-α, Vascular remodeling

Abbreviations: BMP, bone morphogenetic proteins; EndMT, endothelial-to-mesenchymal transition; Exo, exosomes; hUCMSCs, human umbilical cord mesenchymal stem cells; ICAM, intercellular adhesion molecule; LV+S, left ventricular plus septal weight; MCT, monocrotaline; NF-κB, nuclear transcription factor-κB; PAEC, pulmonary arterial endothelial cell; PAH, pulmonary arterial hypertension; PASMC, pulmonary arterial smooth muscle cells; PCNA, proliferating cell nuclear antigen; RVHI, right ventricular hypertrophy index; RVSP, right ventricular systolic pressure; SBP, systemic blood pressure; TEM, transmission electron microscope; TNF-α, tumor necrosis factor alpha; VCAM, vascular cell adhesion molecule; Wnt, Wingless; WT, wall thickness; α-SMA, alpha smooth muscle actin.

These authors contributed equally to this work and should be considered co-first authors.

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1 | INTRODUCTION

Pulmonary arterial hypertension (PAH) is a life-threatening disease, characterized by excessive proliferation of pulmonary vascular cells, eventually leading to pulmonary vascular resistance, vascular remodeling, right ventricular hypertrophy, and heart failure.\(^1,2\) The annual prevalence of PAH exceed 30–50/million individuals every year, for adults, the average age at presentation ranges from 36 to 50 year.

A large number of reports\(^3–5\) showed that excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) is an important pathogenesis in PAH vascular remodeling process. Bone morphogenetic protein type II receptor (BMPR2) is a key factor in the process of PAH pulmonary remodeling.\(^6,7\) BMPR2 levels in pulmonary vasculature are significantly reduced in non-genetic forms of PAH. More than 70% of heritable and 20% of idiopathic PAH cases have the BMPR2 gene mutations.\(^8\) Current available therapies for PAH have limited efficacy, and new therapeutic strategies need to be developed.\(^9\)

Inflammatory is thought to be a powerful trigger factors, promoting the development of PAH in Bmpr2\(^1−/−\) mice.\(^10,11\) The expression of inflammatory markers in clinical PAH patients increased significantly. The inhibitory effects of IL-1 receptor antagonist and antibodies on monocrotaline (MCT)-induced experimental PAH model illustrate the importance of anti-inflammatory treatment. Baicalin, one of several pharmacologically active flavones present in Scutellaria baicalensis Georgi (huang qin), is widely used in traditional Chinese medicinal herbs. Studies\(^12–15\) found that baicalin has several bio-pharmacological effects including antioxidant, anti-inflammatory, antiviral, neuroprotective, anxiolytic, and anti-cancer activities. Recent studies showed that baicalin has therapeutic potential for PAH through inhibiting pulmonary artery pressure and pulmonary vascular remodeling via anti-inflammatory response,\(^15,16\) however, the underlying mechanism remains elusive.

A recent study showed that inflammatory mediator tumor necrosis factor-\(\alpha\) (TNF-\(\alpha\)) can further promote the development of PAH by reducing BMPR2 expression in PASMCs (Yang et al., 2017). Our previous studies have shown that baicalin could inhibit inflammation and improve PASMC vascular remodeling by reducing TNF-\(\alpha\) factor expression and regulation of BMPR2 signaling pathways in MCT-induced PAH rats, respectively.\(^16,17\) However, it has not been illustrated whether the therapeutic effect of baicalin against PAH is associated with TNF-\(\alpha\) regulation of BMPR2 signaling pathways. Therefore, we tested the hypothesis that baicalin inhibits the pulmonary vascular remodeling via regulating the TNF-\(\alpha\)/BMPR2 signaling pathway. These will provide theoretical foundation for the clinical treatment of PAH.

2 | MATERIALS AND METHODS

2.1 | Animals experimental design

Male Wistar rats weighing 200–250 g were purchased from animal center of Second Hospital of Shandong University. Male rats were used to minimize hormonal effects (e.g., of estrogen). The animal protocols followed the guidelines of the Institutional Animal Care and Use Committee (IACUC) of Shandong University. All rats received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health.

Baicalin (purity >95%) was purchased from Sigma and was dissolved in dimethyl sulfoxide (DMSO, Sigma-Aldrich). PAH model was induced by intraperitoneal injection of 50 mg/kg of monocrotaline (MCT) for 6 weeks (Sigma-Aldrich) as per our previously described with modifications.\(^16\) Blood samples were collected at specific time points, the plasma concentration was determined,\(^18\) and we choose the drug concentration according to the report.\(^15\) In the present study, we have tested the lower dose (20 mg/kg, 50 mg/kg) and higher dose (200 mg/kg), the most effective dose is 100 mg/kg, and the main purpose of this present study is to explore the mechanism, so we only choose 100 mg/kg. Baicalin or the same amount of saline solution was given by intragastric administration from 2 days after MCT injection. Forty animals were randomly assigned to four groups: Control, MCT, saline-, and baicalin-treated groups (n = 10 in each).

2.2 | Hemodynamic and the right ventricular hypertrophy assessment

Six weeks later, the rats were anesthetized by isoflurane inhalation (1.5%) and then hemodynamic data were recorded as previously described.\(^16,17\) Briefly, via femoral vein access, an external diameter 0.9 mm polyethylene plastic (PE) catheter was advanced into the pulmonary artery for determination of heart rate (HR), systemic blood pressure (SBP), and right ventricular systolic pressure (RVSP). For assessment of right ventricular hypertrophy, the left ventricle (LV) and plus the septum (LV+S) were harvested, and the weight ratio of the RV to LV+S weight was calculated to quantify the right ventricular hypertrophy. The right ventricular hypertropy index (RVHI) was calculated by the formula: RV/ (LV+S) \(\times 100\).

2.3 | Immunological and immunohistochemical analyses

Post-operation, the lung and heart were quickly harvested and fixed in 4% paraformaldehyde and embedded in paraffin, and the serially sectioned at a thickness of 4–5 \(\mu m\) and then were stained with hematoxylin-eosin (H&E). Evaluation of pulmonary artery structural remodeling, the vascular wall thickness (WT), vascular external diameter (ED), vascular wall area (WA), and total vascular area (TA), WT% (WT/ED), and WA% (WA/TA) was performed as previously study.\(^19\) Fibrosis area was analyzed by Masson's trichrome staining, and then the sections were captured as digital images. The vascular was counted in blind on 30 sections by using a light microscope at a \(\times 400\) magnification. The average of the 10 high-power fields (hpf)
was randomly selected, and positively stained areas were padded with a single color and converted into pixels through optical density (OD) calibration.

Pulmonary artery proliferation was then carried out to analyze the expression of smooth muscle actin (a-SMA) by immunohistochemistry and immunofluorescence. Briefly, after blocking unspecific protein binding with 5% bovine serum albumin for 30 min at room temperature, the lung sections were incubated overnight at 4°C with relevant antibodies. Images were taken with an Eclipse 90i microscope (Nikon). Staining was quantified using Image Pro Plus (IPP) 6.0 image analysis software (Media Cybernetics). All experiments were performed by two examiners blinded to the treatment assignment.

2.4 Cell preparation and culture

Rats PASMC were purchased from Procell Life Science & Technology Co, Ltd., and cultured in special culture medium (Procell) supplemented with 100 Ug/mL of penicillin, 100 IU/mL streptomycin, and 10% fetal bovine serum at 37°C in an incubator. Cells were passaged after 80% confluence, digested with 0.05% trypsin including 0.04% ethylene diamine tetraacetic acid (EDTA, Sigma-Aldrich) in PBS. Recombinant rat TNF-α was purchased from PeproTech (Cat:400-14). Cells were treated with TNF-α (5 ng/mL) for 24 h at 37°C in the presence of baicalin (100 μg/mL) for treatment or an equal volume of DMSO in culture medium group, respectively.

**FIGURE 1** Effect of baicalin on MCT-induced pulmonary hypertension. A comparison of the HR, SBP, RVSP, and RV/LV+S in each group. (B) A comparison of the medial thickness of the pulmonary arterial walls in each group. (C) Hematoxylin and eosin staining. (D) Massons staining. (E) A comparison of the OD value in each group. The data are present as mean ± SD; *p < .05 and **p < .01 compared with Control group; #p < .05 compared with MCT or Saline. Red bar =100 μm. HR, heart rate; MCT, monocrotaline; OD, optical density calibration; RV/LV+S, the ratio of right ventricular weight to left ventricle plus septum; RVSP, right ventricular systolic pressure; SBP, systemic blood pressure; WA%, the percent of vascular wall area (WA)/total vascular area (TA); WT%, the percent of the vascular wall thickness (WT)/vascular external diameter (ED)
2.5 | Cells proliferation and migration assay

PASMC proliferation was measured using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay kit. PASMCs were seeded in 96-well culture plates (1–5×10⁴ cells/well), after 80% confluency, cells were pretreatment with TNF-α for 24 h, and then they were incubated for another 0 h, 24 h, 48 h, and 72 h with baicalin. Following incubation with MTT (5 mg/mL) for 4 h, absorbance was measured at 490 nm.

The migratory function of PASMC was evaluated using a modified Boyden chamber (Transwell; Corning Life Sciences, Inc.) assay with a polycarbonate filter with 8 µm pores placed between the upper and lower chambers. In brief, at 0 h and 24 h following TNF-α pretreatment, cells were treated with baicalin containing 1% FBS and added to the upper chamber. The lower chamber was filled with complete medium in the presence of 10% FBS. After 48 h incubation at 37°C under 5% CO₂, cells that had not migrated were removed, whereas migrated cells were fixed in 4% paraformaldehyde for 10 min at room temperature and stained with the Crystal Violet Staining Solution kit (Solarbio, Beijing Solarbio Science & Technology Co., Ltd.). The number of migrated cells was counted using a Nikon Eclipse 90i microscope.

Quantitative real-time polymerase chain reaction (RT-PCR) analysis was performed using a M×3000P System. 1.5% agarose gel electrophoresis in the presence of ethidium bromide (Sigma-Aldrich) was used to amplification fragments, and β-actin as the internal control. Primers were designed using the Primer Express software package (Applied Biosystems):

- **TNF-α**: 5'-AAATGGGCTCCCTCTATCAGTTC-3' (forward primer) and 5'-TCTGCTTGGTGTTTTGCTACGAC-3' (reverse primer);
- **IL-1β**: 5'-CTTGTTGCAAGTCTTCATGC-3' (forward primer) and 5'-CCCAAGTCAAGGGCTTTGAA-3 (reverse primer);
- **IL-6**: 5'-AAGTCGGAGGCTTAATTACACATGT-3' (forward primer) and 5'-AAGTGCATCATCGTTGTTCATACA-3' (reverse primer);
- **β-actin**: 5'-TCTACAATGAGCTGCGTGTG-3' (forward primer) and 5'-GGTCAGGATCTTCATGAGGT-3' (reverse primer).

The relative gene expression level was determined using the 2⁻ΔΔCT method.

2.7 | Western blot analysis

The protein concentration was detected using a BCA assay kit, lysates were separated by polyacrylamide gel electrophoresis (PAGE) and electrotransferred onto a polyvinylidene fluoride (PVDF), theembranes were blocked in 5% skimmed milk-Tris-buffered saline plus Tween-20 solution, and incubated with primary antibodies, respectively, overnight at 4°C. The primary antibody-labeled membranes were then treated with the horseradish peroxidase (HRP)-conjugated goat anti-rabbit secondary antibody to IgG at room temperature for 1.5 h. The bound antibodies were
visualized by using an enhanced chemiluminescence reagent (Millipore) followed by Bio-Rad Image Lab™. Data were expressed as the relative density of the protein normalized to GAPDH. Primary antibodies of α-SMA (ab21027), BMPR2 (ab170206), NF-κB-p65 (ab16502), p-NF-κB-p65 (ab86299), Smad1/5/8 (sc-6031), p-Smad1/5/8 (sc-12353), ID1 (ab168256), Cyclin D1(MA5-15512), P27Kip1(ab32034), VCAM-1 (ab134047), and ICAM (ab171123) were used, respectively.

2.8 | Statistical analysis

All data are expressed as mean ± SD. Comparisons of parameters between two groups were made with unpaired Student t test. Comparisons of parameters among three groups were made with one-way analysis of variance (ANOVA), followed by the Scheffe post hoc test. Statistical analysis was carried out by using the SPSS 19.0 software. p < .05 was regarded as significant statistical difference.

3 | RESULTS

3.1 | Effect of baicalin on PH vascular remodeling

We evaluated MCT-induced lung and heart injury by detecting RVSP and right ventricular hypertrophy index RV/(LV+S), as shown in Figure 1A. Compared with control group, the RVSP and RV/(LV+S) in
MCT administration group were increased obviously \((p < .05)\), indicating that we successfully established PAH model in rats. However, RVSP and RV/(LV+S) were significantly inhibited in baicalin-treated rats than that in MCT rats \((p < .05)\). However, there was no significant difference in HR and SBP between groups \((p > .05)\).

For histological examination, serial lung sections were stained with H&E and Masson’s trichrome to analyze the medial thickness of pulmonary arterial walls and the degree of fibrosis in vivo. As shown in Figure 1B and C, WT% and WA% of muscular arteries with an external diameter of 15 to 50 µm were significantly increased in MCT group than that in control, but notably decreased in baicalin group \((p < .05)\). The same results were also showed in Figure 1D and E, the Masson’s stained results showed that lung fibrosis was significantly reduced in baicalin group than that in MCT group in vivo \((p < .05)\).

### 3.2 Effect of baicalin on smooth muscle cells

In this study, smooth muscularization cells marker \(\alpha\)-SMA were analyzed by immunohistochemistry and immunofluorescence. The results showed that the expression of \(\alpha\)-SMA was significantly increased in MCT group than that in control, however, that was significantly dropped in baicalin group \((p < .05, \text{Figure 2A–D})\).

### 3.3 Effect of baicalin on inflammatory response

To explore the underlying mechanisms of baicalin against pulmonary vascular remodeling, the mRNA levels of interleukin IL-1\(\beta\) and IL-6 were analyzed by qRT-PCR, and the protein levels of NF-\(\kappa\)B-p65, p-NF-\(\kappa\)B-p65, TNF-\(\alpha\), VCAM-1, and ICAM were detected by western blot. The results showed that the inflammatory factors TNF-\(\alpha\), VCAM-1, ICAM, and the ratio of phosphorylated to total NF-\(\kappa\)B-p65 levels were obviously evaluated in lung tissue when the animals were subjected to MCT, but the inflammatory levels were significantly reduced after treatment with baicalin compared with MCT group \((p < .05, \text{Figure 3A–C})\).

### 3.4 Effect of baicalin on BMPR2 signaling pathway in vivo

BMPR2 signaling plays an important role in the pulmonary vascular remodeling. In the present study, we detected the protein levels of BMPR2, Smad1/5/8, p-Smad1/5/8, and ID1. The results manifested that the levels of BMPR2, Smad1/5/8, p-Smad1/5/8, and ID1 were upregulated remarkably in baicalin group than that in MCT group \((p < .05, \text{Figure 4})\).

### 3.5 Effect of baicalin on PASMC proliferation and migration

The effect of baicalin on proliferation and migration of PASMC were analyzed in vitro. Briefly, cells were pretreated with TNF-\(\alpha\) for 48 h and then were incubated with 100 \(\mu\)g/mL baicalin for 24, 48, and 72 h. MTT assay displayed that the cells viability rate was significantly increased in TNF-\(\alpha\) group compared with the normal groups. After treatment with baicalin, the cells viability rate was significantly decreased \((p < .05, \text{Figure 5A})\).

Transwell assay was performed to observe the effect of baicalin on cells migratory ability. As demonstrated in Figure 5B, the migratory ability was significantly enhanced when the cells were induced by TNF-\(\alpha\) for 48 h, but which was obviously inhibited by baicalin \((p < .05, \text{Figure 5B and C})\).

Furthermore, western blot results showed that the expression of Cyclin D1 was significantly increased, but the P27kip1 was decreased remarkably in MCT group compared with the control. However, baicalin administration could significantly restore these results compared with that in MCT rats \((p < .05, \text{Figure 5D})\).

### 3.6 Effect of baicalin on TNF-\(\alpha\) induces BMPR2 signaling

To further explore the underlying mechanisms, the protein expression of BMPR2 signaling was analyzed by immunofluorescence and western blot in vitro. The results showed that BMPR2, ID1, and
p-Smad1/5/8 were significantly suppressed in TNF-α-induced cells. However, the protein expression was restored when the cells were treated with baicalin (p < .05, Figure 6).

4 | DISCUSSION

Pulmonary arterial hypertension (PAH) is a kind of refractory rare lung diseases, possessing the characteristic of the distal pulmonary arterial remodeling.\textsuperscript{20,21} The pathogenesis of PAH is not very clear, so no effective therapy is available for it. Previous studies\textsuperscript{16,17} suggested that baicalin has potential to inhibit the vascular remodeling in PAH. In the present study, our data confirmed that administration of baicalin could significantly reduce RVSP and RV/LV + S as compared with the MCT-stressed rats (p < .05), warranting baicalin as a novel potential.

Inflammation\textsuperscript{11,22} plays a key role in initiating and maintaining vascular remodeling in PAH animal models. Therefore, to alleviate inflammatory response might attenuate the development of PAH.\textsuperscript{23} Earlier reports have shown that TNF-α level was significantly higher in heritable and idiopathic PAH cases as compared with healthy people.\textsuperscript{11,24,25} In our present study, the results showed significantly decreased mRNA and protein levels of TNF-α, IL-1β, IL-6, and NF-κB in baicalin group than that in MCT-induced PAH group (p < .05). These indicate that baicalin could suppress the inflammatory response in MCT-PAH rats. Therefore, we further analyzed the protein expressions of the adhesion molecules VCAM-1, ICAM, and the ratio of phosphorylated-to-total NF-κB-p65 levels, which were involved in inflammatory processes.\textsuperscript{26,27} Collectively, our results indicated that baicalin attenuated MCT-induced inflammation by decreased expressions of adhesion molecules.

Bone morphogenetic proteins (BMPs) and their receptors are required for PAH-induced right ventricular hypertrophy, which play an important role in the remodeling of pulmonary resistance vessels in the process of PAH.\textsuperscript{10,28,29} BMPR2 was significantly decreased in MCT and chronic hypoxic induced rat PAH...
BMP signaling regulates the occurrence of pulmonary fibrosis process through receptor-mediated phosphorylation and p-Smad1/5/8 transcription factors and alterations in gene transcription. In addition to BMPR2, BMP2, BMP4, BMP6, and BMP9 also have regulatory role for pulmonary vascular cell proliferation. In the present study, we found that the protein expression of BMPR2, Smad1/5/8, p-Smad1/5/8, and ID1 was significantly upregulated by 100 mg/kg baicalin (p < .05). Taken together, these results confirmed that baicalin could significantly repair the MCT-induced PAH pulmonary vascular remodeling through regulation of the BMP signaling pathways.

A recent study showed that the expression of BMPR2 was inhibited by TNF-α in pulmonary vascular cells and pulmonary artery endothelial cells. Many cytokines such as TNF-α, IL-1β, IL-6, and IL-8 are involved in the pathogenesis of PAH. In our study, only TNF-α selectively reduced BMPR2 expression in distal PASMCs and PAECs. On the other hand, TNF-α could promote pulmonary vascular remodeling in the setting of BMPR2 deficiency, increase BMP6 expression, and cause upregulation of transient p-Smad1/5/8 responses in PASMCs.

To further explore the underlying mechanism of baicalin for PAH, we used TNF-α-induced PASMC injury model in vitro. The effect of baicalin on proliferation and migration abilities of PASMC were analyzed. MTT and Transwell results showed that the viability and proliferation rate were significantly increased after cells were treated with TNF-α compared with the normal cells, but baicalin could significantly inhibit the cells proliferation and migration. Furthermore, the PASMC proliferation induced by TNF-α was also observed through detecting the protein expression of Cyclin D1 and P27. Our results showed that the expression of CyclinD1 was significantly higher, P27 was significantly lower in baicalin group compared to that in TNF-α group. Thus, our data provide a strong evidence that baicalin inhibited the PAH vascular remodeling through suppressing the BMPR2 signaling derived by TNF-α.

In summary, the present study demonstrated for the first time that the protective effect of baicalin against PAH vascular remodeling was via regulating the TNF-α/BMPR2 signaling pathway.

**ETHICS APPROVAL AND CONSENT TO PARTICIPATE**

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the US National Institute of Health. Also, all experiments were approved by the Institutional Animal Care and Use Committee of Shandong University. All animals were anesthetized by isoflurane inhalation (1.5%–2%) and then euthanized by cervical dislocation. The work was done in accordance with the Helsinki Declaration’s guidelines.

**CONFLICT OF INTEREST**

All authors declare no conflict of interest.

**DISCLOSURE**

The authors declare that they have no competing interests.

**AUTHOR CONTRIBUTIONS**

Participated in research design: Xue, Zhang, Jiang, Luan.

Conducted experiments: Xue, Zhang, Wang, Li, Xin, Luan.

Contributed new reagents or analytic tools: Xue, Jiang, Qi.

Performed data analysis: Xue, Jiang, Qi, Luan.

Wrote or contributed to the writing of the manuscript: Xue, Zhang, Jiang, Xin, Li, Luan.

**DATA AVAILABILITY STATEMENT**

All data generated in this study are included in this manuscript.

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