

Baicalin prevents pulmonary arterial remodeling in vivo via the AKT/ERK/NF- κ B signaling pathways

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Abstract

Pulmonary arterial hypertension is a rapidly progressive and often fatal disease. As the pathogenesis of pulmonary arterial hypertension remains unclear, there is currently no good drug for pulmonary arterial hypertension and new therapy is desperately needed. This study investigated the effects and mechanism of baicalin on vascular remodeling in rats with pulmonary arterial hypertension. A rat pulmonary arterial hypertension model was constructed using intraperitoneal injection of monocrotaline, and different doses of baicalin were used to treat these rats. The mean pulmonary arterial pressure (mPAP) and right ventricular systolic pressure (RVSP) were measured with a right heart catheter. Moreover, the hearts were dissected to determine the right ventricular hypertrophy index (RVHI). The lung tissues were stained with H&E and Masson's staining to estimate the pulmonary vascular remodeling and collagen fibrosis, and the expression of proteins in the AKT, ERK, and NF- κ B p65 phosphorylation (p-AKT, p-ERK, p-p65) was examined by Western blot analysis. We found that compared with untreated pulmonary arterial hypertension rats, baicalin ameliorated pulmonary vascular remodeling and cardiorespiratory injury, inhibited p-p65 and p-ERK expression, and promoted p-AKT and p-eNOS expression. In conclusion, baicalin interfered with pulmonary vascular remodeling and pulmonary arterial hypertension development in rats through the AKT/eNOS, ERK and NF- κ B signaling pathways.

Keywords

pulmonary arterial hypertension, baicalin, vascular remodeling, proliferation, inflammation

Date received: 6 April 2019; accepted: 5 September 2019

Pulmonary Circulation 2019; 9(4) 1–10

DOI: 10.1177/2045894019878599

Introduction

Pulmonary arterial hypertension (PAH) is a commonly found disease with a high rate of disability and mortality. Seventy-five percent of PAH patients die within five years after being diagnosed, and those with right heart failure die within one year on average. So far, there is no method for preventing the occurrence of PAH.^{1,2} Pulmonary vascular remodeling is the pathological basis of PAH, and it is the target of many clinical medicines, of which the mechanism and intervention strategy have received increasingly more attention recently.³ A previous study has shown that pulmonary arterial smooth muscle cell (PASMC) damage-induced inflammation activated proliferation-related

signaling pathways, including phosphatidylinositol-4, 5-bisphosphate 3-kinase (PI3K)/AKT, mitogen-activated protein kinase (MAPK)/ERK1/2 and NF- κ B p65, which further led to increased PASMC proliferation, migration and differentiation, and decreased apoptosis.^{4,5} Consequently, the pulmonary small vessel wall became thicker, vessel stenosis was formed and the extracellular matrix was greatly increased. With the increased knowledge of PAH

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development, many target drugs have been explored, including prostaglandins, PDE-5 inhibitor, and endothelin-receptor antagonist.^{6,7} These drugs improve to some extent the clinical symptoms of PAH patients; however, they cannot reverse the pulmonary vascular remodeling process and prevent PAH development.⁸ For example, sildenafil was demonstrated to inhibit pulmonary vascular remodeling and approved for treatment of PAH by the Food and Drug Administration (FDA) in 2005, but there are many adverse reactions and side effects.^{9–11}

MAPK cascade activation plays center roles in many signal pathways, it receives membrane receptor exchanged and transferred signal and then sends into cell nucleus, and it shows key roles in many cell proliferation-related signals.^{12,13} MAPK stays in stable condition regularly; however, when cells are activated by growth factors or other reasons, MAPK could receive the MKK and MKKK activating signal and become phosphorylated successively.¹⁴ In mammals, MAPK/ERK exists extensively in many tissues regulating the cell proliferation and differentiation. PI3K/AKT pathway is a core regulator of cell metabolism, growth, and survival. Some pre-clinical evidences demonstrate the efficacy and safety of its inhibitors in Biliary tract cancers,¹⁵ and another plantamajoside inhibited NF- κ B activation and inflammatory response through suppressing PI3K/AKT pathway in LPS-stimulated human gingival fibroblasts.¹⁶

Baicalin is a flavonoid compound isolated from the root of *Scutellaria baicalensis*, which shows wide bioactivities including diuresis, bacteriostasis, anti-inflammation, spasmolysis (inhibition of VSMC proliferation), and anti-cancer. Furthermore, it plays important roles in clinical and basic research.¹⁷ Moreover, it eliminates reactive oxygen species, absorbs UV, and inhibits melanogenesis.¹⁸ In the cardiovascular diseases field, increasingly more attention has been paid to the application of baicalin; however, there are only few reports on the use of baicalin for PAH. Herein, we investigated the effects and mechanism of baicalin in pulmonary arterial remodeling and PAH development.

Methods

Animals

Sixty male specific pathogen-free (SPF) Sprague Dawley (SD) rats weighing 200 ± 20 g were provided by Guangdong Medical Animal Experimental Center. The animal protocols followed the guidelines of the Institutional Animal Care and Use Committee of Guangdong Medical University, and the experiments were conducted according to the National Institutes of Health (NIH) Guide for the care and use of animals in laboratory experiments.

Animal culture and experimental grouping

Sixty eight-week-old healthy male SPF SD rats were randomly divided into six groups: control, PAH, low-dose

baicalin (20 mg/kg), medium-dose baicalin (100 mg/kg), high-dose baicalin (200 mg/kg), and sildenafil positive control (50 mg/kg). Each group had 10 rats.

PAH rat model construction and intervention

According to a previously described method,¹⁹ rats were weighed and intraperitoneally injected with monocrotaline (MCT) at 50 mg/kg. The control group rats were injected with an equal volume of saline solution. Both control and PAH rats were injected with 1.5 ml of saline solution for another 29 days, while the low-dose baicalin group (20 mg/kg), medium-dose baicalin group (100 mg/kg), high-dose baicalin group (200 mg/kg), and sildenafil positive control group (50 mg/kg) were injected with the corresponding medicine for 29 days. The feeding conditions, breathing, weight, and morbidity rate of rats in each group were recorded.

Tissue specimen preparation

Firstly, the rats were humanely euthanized by exsanguination under anesthesia, and their survival was monitored at the time. Rats were tested for pulmonary artery pressure (PAP) and killed, and then the thoracic cavity was opened to obtain the heart and lung tissues. The pulmonary marginal tissue was excised and fixed in 4% formaldehyde solution for 24 h, washed with cold saline solution, and prepared as 5- μ m paraffin-embedded sections, which were dyed with Masson's, hematoxylin and eosin (H&E), and immunofluorescence histochemistry (IHC) staining. The rest of the lung tissue was snap frozen in liquid nitrogen and kept in -80°C for other experiments.

Right ventricular hypertrophy index detection

The cardiac vessels and atrium were removed following the atrioventricular groove direction, and then left ventricle (LV)+interventricular septum (IS) and right ventricle (RV) were isolated through the interventricular septum and washed with cold saline solution repeatedly to clean away the remaining blood. The liquid on the surface was sucked by filter paper and each part was weighed by electronic scales. Then, the right ventricular hypertrophy index (RVHI) was calculated by the following equation: $\text{RVHI} = \text{RV}/(\text{LV}+\text{IS})$.

Pulmonary arterial pressure and right ventricular systolic pressure

After medicine intervention for 30 days, rats were weighed and anesthetized by intraperitoneal injection of 7% chloral hydrate at 5 ml/kg. Rats were fixed in the supine position on the operating table. A polystyrene microtubule was moistened completely in heparin saline before being connected with a pressure transducer and Medlab physiological

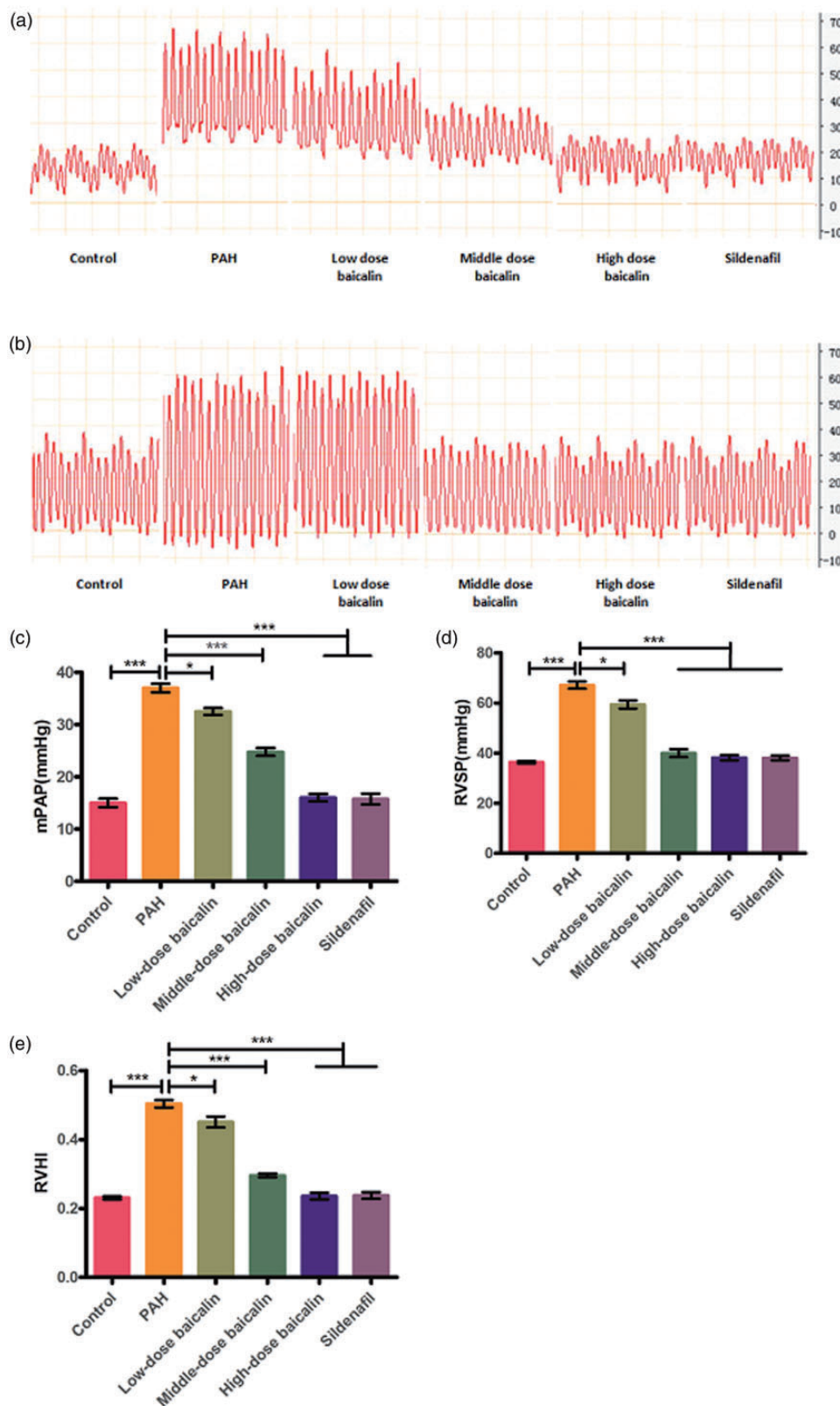


Fig. 1. The effects of baicalin treatment on PAH. (a) The mPAP of rats in the every experimental group; (b) The RVSP of rats in the every experimental group; (c) Difference analysis of mPAP from different groups of rats; (d) Difference analysis of RVSP from different groups of rats; (e) Difference analysis of RVHI from different groups of rats. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

recorder. At the same horizontal level, the machine was set to zero. The right side external carotid vein of the rats was carefully isolated and a “V” cut was shaped at the vein lateral wall by ophthalmic scissors, and then a microtubule was inserted through the vessel until it reached the right side external carotid vein and advanced proximo-distally. With the waveform variation displayed on the Medlab physiological recorder, the position of the microtubule could be identified. The mean PAP (mPAP) and right ventricular systolic pressure (RVSP) values of rats in each group were also recorded.

Histopathology

The marginal region of the lung tissue was dissected and fixed in 10% neutral formalin solution for 16–24 h, and then embedded in paraffin and cut into 4- μ m sections for histologic examination. The morphological changes of the lung tissue were observed by H&E staining. Five pulmonary arterioles with an external diameter of 50–150 μ m were randomly selected under a light microscope at 200 \times magnification, and then the pulmonary arterioles lumen area and thickness were measured; wall area (WA) and wall thickness (WT) were calculated as $WA = 1 - \text{lumen area}/\text{vessel total area}$, $WT = 1 - \text{vessel diameter}/\text{external diameter}$.

The degree of lung tissue fibrosis was estimated by Masson's trichrome staining of collagen deposition. The paraffin sections were dewaxed and dyed with Weigert iron hematoxylin, followed by ponceau acid fuchsin staining, and redyed with aniline blue staining after being treated with phosphomolybdic acid. The sections were treated with 1% glacial acetic acid and dehydrated with 95% ethanol followed by 100% ethanol, and then rinsed with dimethylbenzene and sealed with neutral balsam. Each section was observed under a light microscope at 400 \times magnification. Five fields of each section were observed and imaged to determine the lung tissue fibrosis degree, which was measured by a CDD camera under a microscope (ECLIPSE 80i, Nikon, Tokyo, Japan).

IHC analysis

After being taken off paraffin wax with xylene, the paraffin-embedded lung tissue sections were handled with different concentrations of alcohol and repaired for three times in PBS solution. The sections were incubated overnight at 4 $^{\circ}$ C in a wet box by adding PBS with the first antibodies (CD31: 1:500 or α -SMA: 1:500), and then the tissues were covered by the secondary antibody by dropping with corresponding horse radish peroxidase (HRP) markers (CY3: 1:500). After microwave treatment in the citric acid antigen repair buffer (pH6.0), the tissues were incubated with another first antibody (AKT phosphorylation (p-AKT): 1:1000) and corresponding HRP markers (FITC: 1:500). The sections were dried and treated by spontaneous fluorescence quencher for 5 min, and subsequently the interphase

nucleus counter stained with 4',6-diamidino-2-phenylindole (DAPI) showed blue. At last, these sections were observed and taken pictures under the laser confocal microscope (FV3000; Olympus).

Western blot analysis

Total protein was extracted from the lung tissues using RIPA lysis buffer (Beyotime Institute of Biotechnology, Nanjing, China) supplemented with protease inhibitors. The concentration was determined by the bicinchoninic acid protein assay. Equal amounts (10 μ g) of protein extracts were electrophoresed in SDS-polyacrylamide gels. Proteins were blotted onto PVDF membranes by electrophoretic transfer and incubated with primary antibodies against p65 phosphorylation (p-p65) (1:1000), ERK phosphorylation (p-ERK) (1:1000), ERK (1:1000), p-AKT (1:1000), AKT (1:1000), eNOS phosphorylation (p-eNOS) (1:1000), eNOS (1:1000) and α -Tubulin (1:1000), followed by incubation with HRP-conjugated secondary antibodies (1:10,000 dilution). Super ECL plus was used to visualize the protein bands, which were analyzed by Quantity one software. α -Tubulin was used as an internal reference.

Statistical analysis

The results are expressed as mean \pm SEM. The *t*-test was used for comparisons of two groups, and ANOVA was used for multi-group comparisons. Newman-Keuls test was used for two-sample comparisons among groups. The data were analyzed by GraphPad Prism 5.0 statistical software.

Results

Measurement of physiological indexes associated with PAH development

The pressure curves of the rat right atrium, right ventricle, and pulmonary artery recorded by a Medlab physiological recorder are shown in Fig. 1. The results showed that mPAP and RVSP in the PAH group were higher than those in the control group ($P < 0.001$). However, in the medium- and high-dose baicalin groups, and in the sildenafil group, the mPAP and RVSP were significantly lower compared with the PAH group ($P < 0.001$), and the effect was dose-dependent.

The RVHI of the PAH group was obviously higher than that of the control group ($P < 0.001$). However, in the medium- and high-dose baicalin groups, and in the sildenafil group, the RV/LV+IS was significantly lower compared with the PAH group ($P < 0.001$), in a dose-dependent manner.

H&E staining of pulmonary small vessels

After H&E staining of pulmonary small vessels, the wall thickness of pulmonary arterioles with 50–200 μ m external

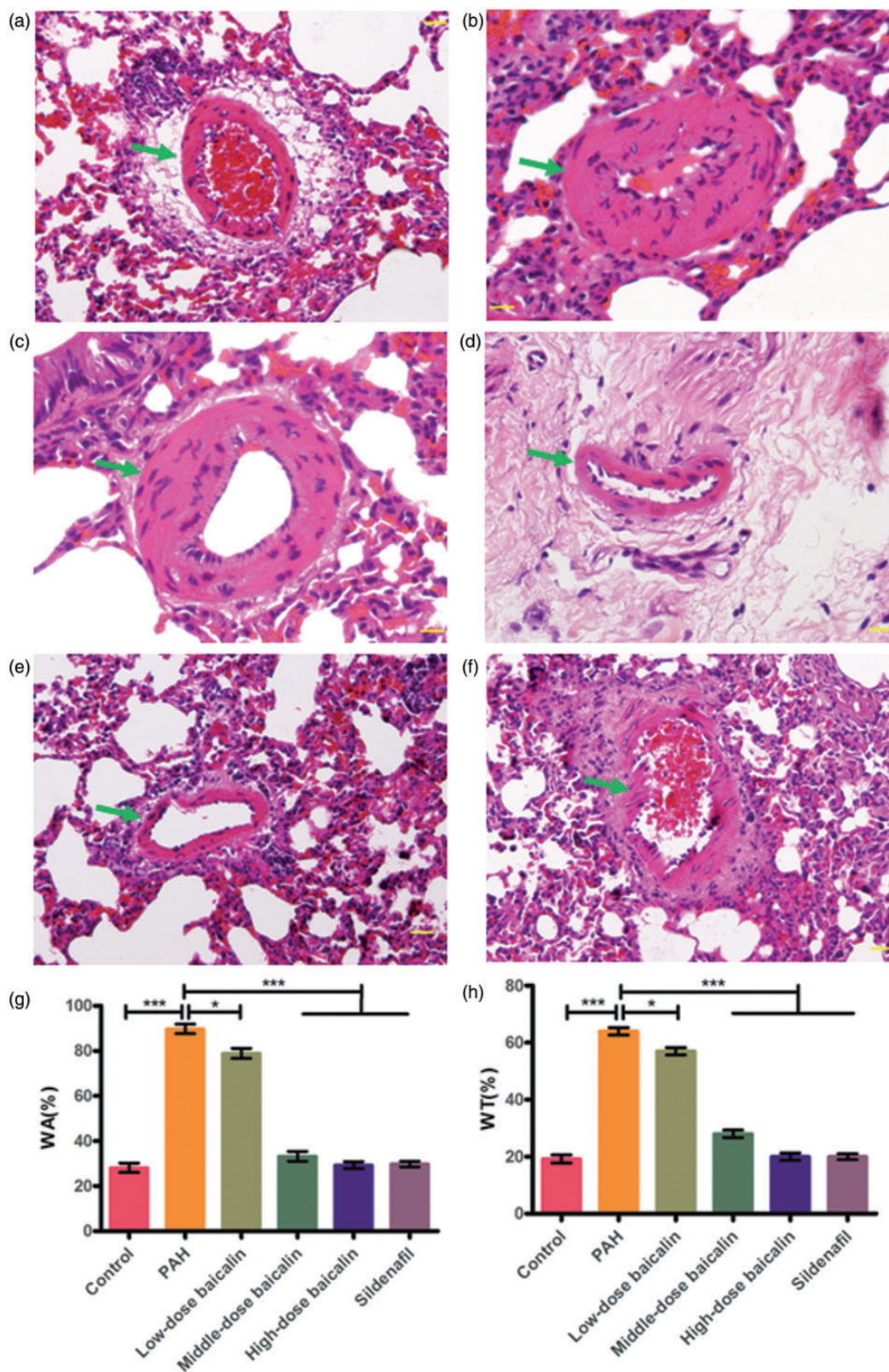


Fig. 2. The pulmonary small vessels and vascular remodeling of rat by H&E staining. (a) Staining in the control group; (b) Staining in the PAH group; (c) Staining in the low-dose baicalin group; (d) Staining in the medium-dose baicalin group; (e) Staining in the high-dose baicalin group; (f) Staining in the sildenafil group; (g) WT index of rats in each group. (h) WA index of rats in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

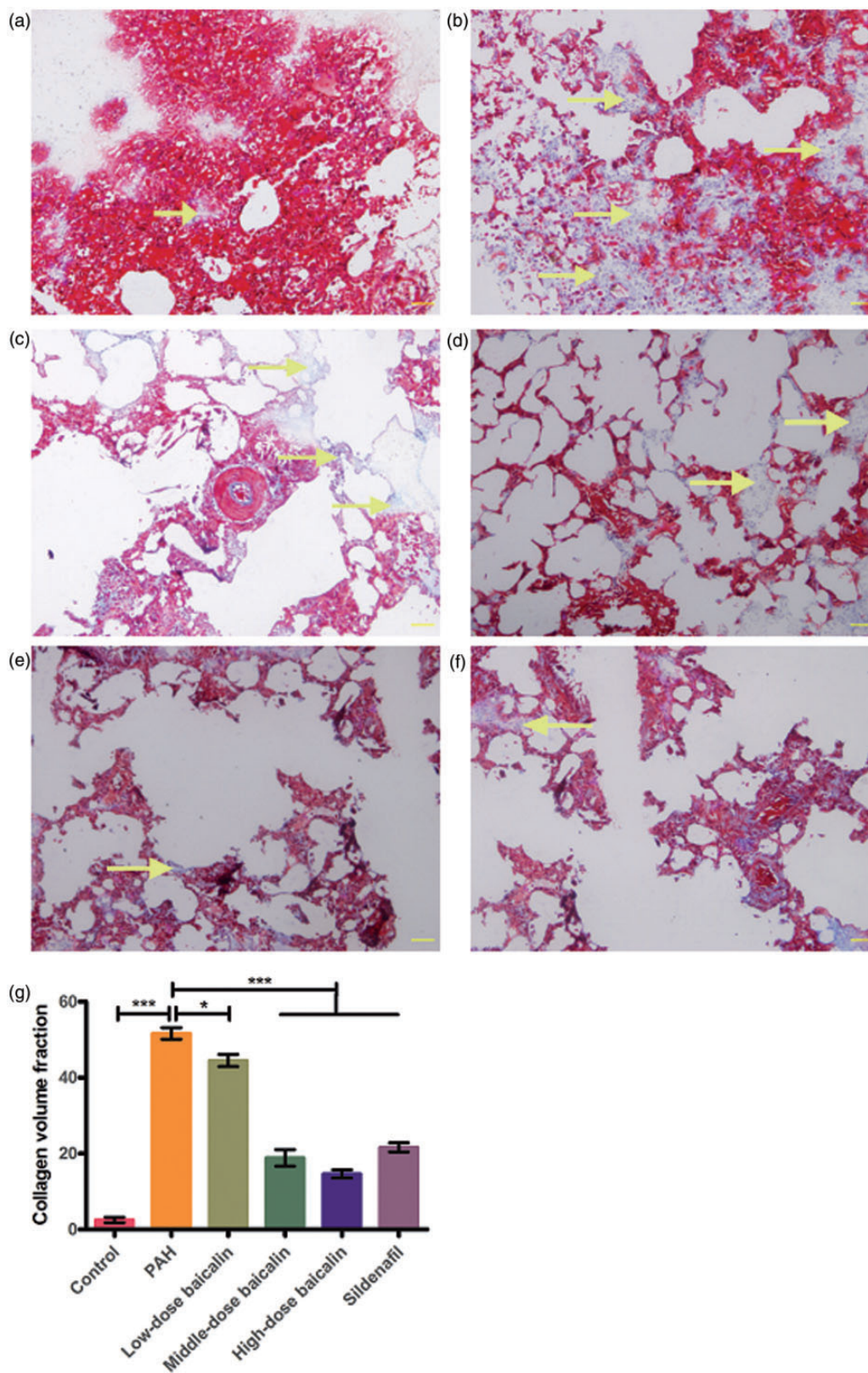


Fig. 3. The lung tissue fibrosis of PAH rat by Masson's trichrome staining. (a) Staining in the control group; (b) Staining in the PAH group; (c) Staining in the low-dose baicalin group; (d) Staining in the medium-dose baicalin group; (e) Staining in the high-dose baicalin group; (f) Staining in the sildenafil group; (g) Volume of collagen fiber in the lung tissue of each experimental group (%). * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

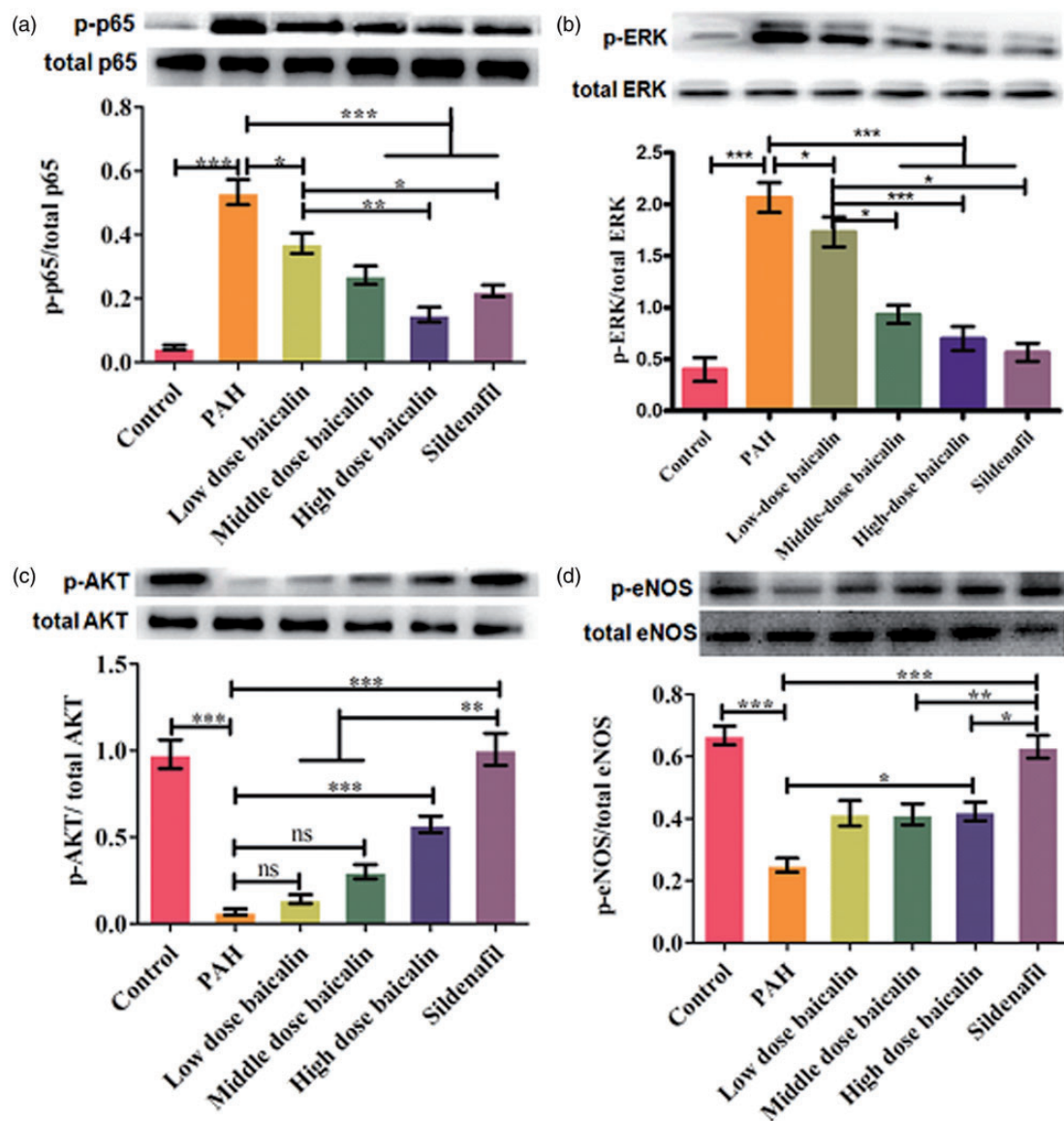


Fig. 4. The expression and phosphorylation levels of related proteins. (a) p-p65/total p65 in each group; (b) p-ERK/total ERK in each group; (c) p-AKT/total AKT in each group; (d) p-eNOS/total eNOS in each group. * $P < 0.05$, ** $P < 0.01$, *** $P < 0.001$.

diameter was measured under optical microscopy at $200\times$ magnification. Pulmonary arterioles of each experimental group were quantitatively measured by image software and the results are shown as follows: in the PAH group, the WT and WA indexes were significantly higher compared with the control ($P < 0.001$). The medium-dose and high-dose baicalin groups, and the sildenafil group had decreased WT and WA indexes compared with the PAH group. The baicalin effect was dose-dependent ($P < 0.001$) (Fig. 2).

We found that in the PAH group, the pulmonary arteriole wall was significantly thickened, and strait lumen was observed. In contrast, PAH rats in the medium-dose and high-dose baicalin groups, and in the sildenafil group, had increased pulmonary arteriole lumen and decreased wall thickness.

Masson's staining of lung tissues

The degree of lung tissue fibrosis was estimated by Masson's trichrome staining of collagen deposition (Fig. 3). We found that the lung tissue of PAH group rats had wide and multifocal collagen deposition. However, PAH rats treated with medium-doses or high-dose baicalin, or with sildenafil showed improved collagen deposition in the lung tissue and right ventricle. The baicalin effect was dose-dependent ($P < 0.001$).

Western blot analysis of rat lung tissue

The p-p65 and p-ERK/ERK protein expression levels in the lung tissue of PAH group rats were significantly higher than those in the control group ($P < 0.001$). However, PAH rats

treated with a medium-dose or high-dose of baicalin, or sildenafil showed lower p-p65 and p-ERK/ERK protein expression levels compared with those in the untreated PAH group, and the baicalin effect was dose-dependent ($P < 0.001$) (Fig. 4a and b).

By contrast, the expression levels of p-AKT and p-eNOS of the PAH group were remarkably lower than those of the control group ($P < 0.001$). Baicalin promoted to phosphorylate AKT and eNOS with the increasing of treated concentration, and the high-dose baicalin up-regulated their expression to the highest level and approached the sildenafil treatment (Fig. 4c and d).

IHC analysis of p-AKT in pulmonary arterioles

The location and expression of p-AKT protein were investigated in PASMCs and endothelial cells by IHC analysis, respectively. The results showed p-AKT to express in the endothelial cells. Intriguingly, AKT phosphorylation was low in the pulmonary arterioles from PAH group, and furthermore the phosphorylated level increased with the dosage of baicalin (Fig. 5), which shared the same trend with the above Western blot analysis.

Discussion

Vascular remodeling leads to PASMC proliferation and apoptosis inhibition, resulting in vascular medial smooth muscles getting thicker, which plays important roles in PAH development.²⁰ In the present study, we documented that baicalin prevented significantly MCT-induced PAH, pulmonary vascular remodeling and tissue fibrosis, and the protective mechanism may be through activation of AKT/ERK/NF- κ B signaling pathway and phosphorylation of eNOS protein (Fig. 6). These provided the novel evidences for the effect and mechanism of baicalin on experimental PAH, holding promise in improving this class of medications palliative therapy agents.

PASMC proliferation and pulmonary vascular remodeling are used as a modulator of PAH development and provide the therapeutic target of the disease, and hence novel effective agents and more approaches remain urgently needed for the treatment currently.²¹ In this study, MCT was used to establish a rat PAH model, which is widely regarded as classical research method of PAH, especially in the field of drug and pharmacology.²² The pathological process of this disease model includes induction of chronic inflammation, which leads to the proliferation and migration of PASMCs, and the transformation of endothelial cells, leading to remodeling of the pulmonary artery and an increase in pulmonary artery pressure.^{19,23} After baicalin intervention, medium and high doses of baicalin significantly reduced the mPAP and RVSP in rats. H&E staining of the lung tissue showed that medium and high doses of baicalin significantly inhibited pulmonary vascular remodeling. Furthermore, Masson's trichrome staining of the

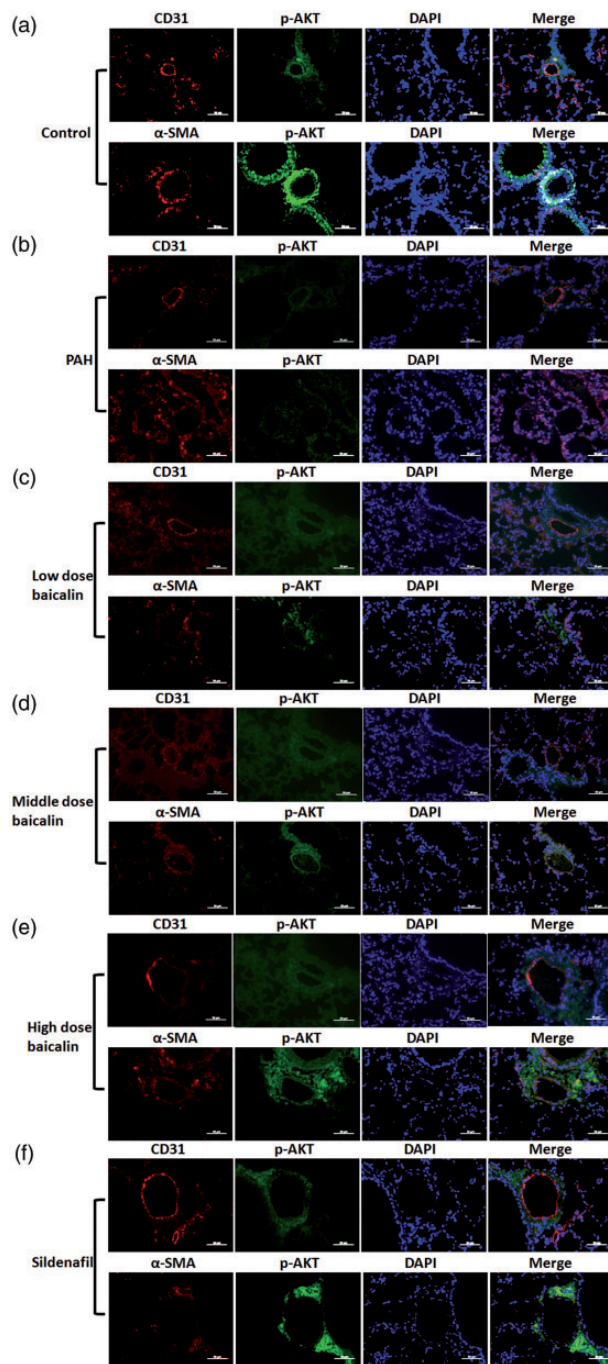


Fig. 5. The location of p-AKT protein in PASMCs and endothelial cells by IHC staining. (a) Staining in the control group; (b) Staining in the PAH group; (c) Staining in the low-dose baicalin group; (d) Staining in the medium-dose baicalin group; (e) Staining in the high-dose baicalin group; (f) Staining in the sildenafil group.

lung tissue showed that medium and high doses of baicalin significantly inhibited collagen fibrosis in the lung tissue. The RVHI of rats after intervention was also analyzed, showing that the medium and high doses of baicalin significantly reduced this index. Taken together, our results indicate that baicalin at medium and high doses can indeed alleviate pulmonary hypertension pressure, pulmonary

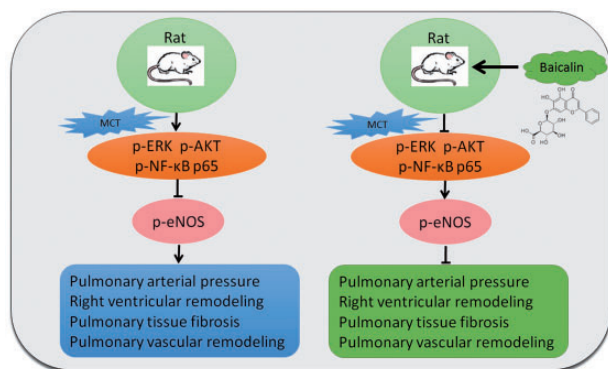


Fig. 6. Baicalin inhibits pulmonary arterial remodeling in vivo via the AKT, ERK, and NF- κ B signaling pathways.

vascular remodeling, and pulmonary fibrosis in rats, in a dose-dependent manner. The high dose of baicalin had a similar effect as sildenafil, which was used as a positive control.

Recently, the pharmacological mechanism of baicalin on MCT or hypoxia-induced PAH was reported to associate with inflammatory response, HMGB1/RAGE, and MMP-9 signaling pathways.^{24–26} We also found that baicalin inhibited proliferation and inflammation by inhibiting expression and phosphorylation of ERK and p65 signaling pathways, thereby preventing pulmonary vascular remodeling and PAH. Zhang et al.²⁷ confirmed baicalin to have a therapeutic effect on PAH via inhibition of NF- κ B signaling to further activation of BMP family, and nevertheless we demonstrated further that baicalin inhibited phosphorylation of ERK and p65 signaling in concentration-dependent manner. Notably, the phosphorylated level of p65 protein in the group of the high-dose baicalin was even lower than that from positive control sildenafil. Moreover, relaxing blood vessels was achieved by activating the AKT/eNOS signaling pathway. Unlike the previous study suggesting that baicalin attenuated hypoxic PAH by up-regulating the AKT/HIF-1 α /p27-associated pathway,²⁸ we found baicalin to phosphorylate AKT and eNOS with the increasing of concentration and especially p-eNOS contributed to the functional changes of pulmonary vascular tissues. Pulmonary artery endothelial cell proteins and lung tissue proteins may play a major role in reducing pulmonary artery pressure. Interestingly, AKT1 was required for the development of PAH in mice and PSMCs in small remodeled pulmonary arterioles had hyper-phosphorylation of AKT^{29,30}; however, the present results revealed AKT phosphorylation to mainly express in the endothelial cells. This study showed that baicalin suppressed pulmonary arterial remodeling and PAH development in vivo via the AKT/ERK/NF- κ B signaling pathways, and thus it is a promising therapeutic strategy for PAH.

Authors' contributions

GY and JW was the major contributor of the presented work. GY, JW, TY and HG performed the experiments and collected the data.

GY, JW, YH and WL analyzed the data and made the graphs. YH, JW, XS and WL performed the statistical analysis and revision of manuscript. GY, XS and WL wrote the manuscript and revised the graphs. WL, YH and SH designed this study. This work was performed in the guidance and lab of WL, ZW, JC and SH.

Conflict of interest

The author(s) declare that there is no conflict of interest.

Funding

This paper was supported by National Natural Science Foundation of China (no. 81970056, 81741129, 81700269), Collaborative Innovation and Platform Environment Construction Projects of Guangdong Province (2015A050502049), Natural Science Foundation of Guangdong Province (2015A030313520 and 2016A020214017) and Characteristic Innovation Project of Department of Education of Guangdong Province (2016KTSCX048).

Ethical approval

The animal protocols followed the guidelines of the Institutional Animal Care and Use Committee of Guangdong Medical University, and the experiments were conducted according to the National Institutes of Health (NIH) Guide for the care and use of animals in laboratory experiments.

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