Protective effect of baicalin on experimental pulmonary arterial hypertension through inhibition of pulmonary vascular remodeling

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Abstract: Previous studies have shown that baicalin can attenuate pulmonary arterial hypertension and right ventricular hypertrophy. However, the potential mechanism remains unexplored. Nuclear factor-κB (NF-κB) and bone morphogenetic protein (BMP) signaling pathway play an important role in monocrotaline (MCT) induced pulmonary arterial hypertension (PAH). Therefore, we aimed to observe the regulation of baicalin on the NF-κB-BMP axis and the subsequent anti-proliferation in pulmonary vascular. Our results showed that baicalin could significantly decrease right ventricular systolic pressure (RVSP) and the RV/left ventricle plus septum ratio ($P < 0.05$), and attenuate vascular remodeling. Furthermore, the result of westen blot showed that the protein expression level of BMP receptor 2 (BMPR2) was significantly increased, while NF-κB p65, p-NF-κB p65, inhibitor of NF-κB (I-κBα) and the BMP antagonist, gremlin 1 were significantly down-regulated in the baicalin group ($P < 0.05$). On the other hand, the result of immunohistochemical staining in lung showed that the capillary density of pulmonary arterioles significantly increased in the baicalin group compared with the MCT group ($P < 0.05$). We concluded that baicalin exerted the protective effects against the lung and heart damage through inhibiting NF-κB-BMP signaling pathway, providing new mechanistic information about PAH and right ventricular hypertrophy.

Keywords: Baicalin; Pulmonary arterial hypertension; Pulmonary vascular remodeling; NF-κB; BMP

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1. Introduction

Pulmonary arterial hypertension (PAH) is characterized by vascular obstruction caused a sustained elevation of pulmonary vascular resistance, vascular remodeling, and right ventricular hypertrophy and failure[1,2]. The pathogenesis of PAH is complex, including pulmonary arterial endothelial cell dysfunction and pulmonary arterial smooth muscle cell (PASMCs) proliferation[3]. It's well known that exuberant pulmonary vascular remodeling (PVR) is a key process of PAH[4], which is mainly caused by high pulmonary blood flow[5]. Neointimal formation and hyperplasia of the medial vascular wall are attributed to an imbalance between proliferation and apoptosis of PASMCs[6,7]. Although there are a large number of treatment options for PAH so far, including inhaled nitric oxide, vasodilators, calcium channel blockers, intravenous prostacyclin, and endothelin receptor antagonists, most patients eventually become resistant to therapy and succumb to the disease. Therefore, novel approaches are urgently required for the treatment of PAH.

Nuclear factor-κB, an inflammation-related transcription factor, plays an important role in PVR, and regulation of NF-κB pathway can promote vascular intima formation, smooth muscle cell proliferation and remodeling[8]. Its activation can be inhibited by ubiquitin-proteasome

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system through reducing the degradation of I-κBa[9]. Recent few reports have advocated that MCT-treated PAH-induced right ventricular hypertrophy is attenuated in cardiac-specific IκBα triple mutant transgenic mice compared with wild-type mice, suggesting a protective role of NF-κB in right ventricular hypertrophy[10]. Furthermore, the cell apoptotic and epithelial-mesenchymal transition (Endo-MT) events caused by NF-κB signaling pathways in the lungs have significant correlation with bone morphogenetic protein (BMP) pathway[11]. Our previous study has shown that baicalin can protect MCT-induced lung and heart injury through inhibiting the inflammatory reaction, and down-regulating the NF-κB signaling pathway. However, the basic mechanism has not been fully understood[12].

Baicalin, a flavonoid compound purified from the dry roots of *Scutellaria baicalensis* Georgi (huang qin), has been shown to possess several biological effects[12–15]. Previous studies have revealed that baicalin attenuates pulmonary artery pressure, reduces right ventricular hypertrophy, and attenuates PVR in MCT-induced PAH *in vivo via* anti-inflammatory response and inhibition NF-κB pathways[11,16]. In recent years, several reports have shown that baicalin can inhibit the proliferation of human cancer cells and PAMSC proliferation, including antiproliferation and differentiation, and increase the sensitivity to apoptosis[13,17,18]. However, the potential mechanism of baical in the prevention of cardiovascular diseases remains unexplored. In the present study, we tested the hypothesis that baicalin protected the lung and heart damage through inhibiting NF-κB-BMP signaling pathway, and the subsequent and endothelial to mesenchymal transition (EMT) events in the lungs were also discussed.

2. Materials and methods

2.1. Animals

All animals received humane care in compliance with the Guide for the Care and Use of Laboratory Animals published by the U.S. National Institute of Health (NIH Publication No. 85-23, revised 1996). The study protocol was approved by the Institutional Animal Care and Use Committee (IACUC) of the Second Hospital of Shandong University.

2.2. Experimental protocols

Baicalin (purity > 95%) was purchased from Sigma (St. Louis, MO, USA) and dissolved in dimethyl sulfoxide (DMSO). The PAH model was induced by intraperitoneal injection of 60 mg/kg MCT (Sigma-Aldrich, USA) as previously described with modifications[11]. A total of 50 animals were randomly assigned into five groups as follows: control, baicalin, PAH and PAH + baicalin groups (n = 10 in each). Baicalin group was given baicalin 100 mg/kg by intragastric administration. At 4 weeks after MCT injection when severe PAH was established, rats were anesthetized with pentobarbital (30 mg/kg, *i.p.*, Sigma Aldrich) and inserted with a 3F-Miller micro tip catheter *via* the right jugular vein into the right ventricle (RV) to obtain the right ventricular systolic pressure (RVSP). Results of the hemodynamic parameters, right ventricular hypertrophy, and pulmonary arterial pathological changes were used to evaluate whether the model of PAH was successfully established.

2.3. Immunological and immunohistochemical analyses

The rats were sacrificed after hemodynamic measurements, and the lung and heart were quickly harvested, fixed *in situ via* the trachea cannula with buffered 4% formaldehyde, and embedded in paraffin. The sections were cut into 4–5 μm slices and stained with streptavidin peroxidase and hematoxylin and eosin (H&E). To evaluate PVR, the vascular wall thickness (WT), vascular external diameter (ED), vascular wall area (WA) and total vascular area (TA) were measured to calculate WT% (= WT/ED) and WA% (= WA/TA).

2.4. RNA extraction and quantitative real-time polymerase chain reaction (qRT-PCR)

Total RNA was extracted using RNeasy kit (Qiagen, Valencia, CA), and reverse transcription was performed using iScript cDNA synthesis kit (Bio-Rad, Hercules, CA) according to the manufacturer's instructions. qRT-PCR analysis was performed to detect the relative pulmonary expression levels of CD31, α-smooth muscle actin (α-SMA) and E-cadherin using gene-specific primers as previously described. Data were analyzed using the ABI Prism 7900 sequence detection system software (version 2.2), and β-actin was used as an internal control for input RNA.

2.5. Western blotting analysis

Lung tissue was pulverized in liquid nitrogen, and cytosolic and nuclear proteins were extracted using NE-PER nuclear and cytosolic extraction reagents (Pierce). Protein extraction buffer and equal amounts of protein were denatured and separated by sodium dodecyl sulfatepolyacryl-amide gel electrophoresis (SDS-PAGE). Protein concentrations were assessed using the BCA Protein Assay kit (Santa Cruz Biotechnology). Briefly, 10 μg of total protein was electrophoresed on 4%–20% gradient SDS-PAGE gels and transferred onto a nitrocellulose membrane. Western blotting and the subsequent quantification of each blot were performed as described previously. The primary antibodies used in this study included BMPR2 (ab170206), IκB-α (ab76429), NF-κB p65 (ab16502), p-NF-κBp65 (ab86299), and gremlin (sc-18274).

2.6. Statistical analysis

All experiments were performed at least three times for each determination. Data were 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* multiple comparison test. Statistical analysis was carried out by using the SPSS 13.0 software. $P < 0.05$ was regarded statistically significant.

3. Results

3.1. Effect of baicalin on pulmonary artery blood flow and right ventricular hypertrophy

The right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index [RV/(LV+S)] were used to evaluate the model of MCT-induced PH. The results showed that RVSP and RV/LV+S were significantly increased in MCT-induced PAH rats compared with the control rats, confirming that injection of MCT led to severe PAH. However, such elevation was significantly inhibited by the application of baicalin ($P < 0.05$, Fig. 1). These results indicated that the baicalin treatment could inhibit the damage of lung in MCT-induced PAH rats.

3.2. Effect of baicalin on PVR and right ventricular hypertrophy

To evaluate PVR, lung sections were stained with H&E and examined by light microscopy to detect the medial thickness of the pulmonary arterial walls. As shown in Figure 2, WT% and WA% of muscular arteries were significantly increased in the MCT-induced PAH group compared with the controls, while they were notably decreased in the baicalin group ($P < 0.05$).

3.3. Effect of baicalin on pulmonary vessel density and EMT in lung

The capillary density of pulmonary arterioles was detected by immunohistochemical staining (CD31) in

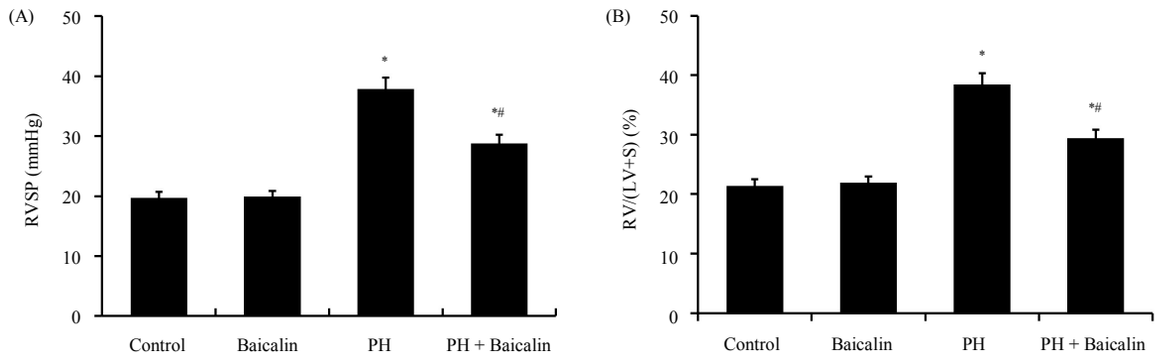

Figure 1. Effect of baicalin on MCT-induced pulmonary artery hypertension. A comparison of the right ventricular systolic pressure (RVSP) in each group. (B) A comparison of the ratio of right ventricular weight to left ventricle plus septum [RV/(LV+S)] % in each group. $^{*}P < 0.05$, *vs.* control; $^{\#}P < 0.05$, *vs.* PH group.

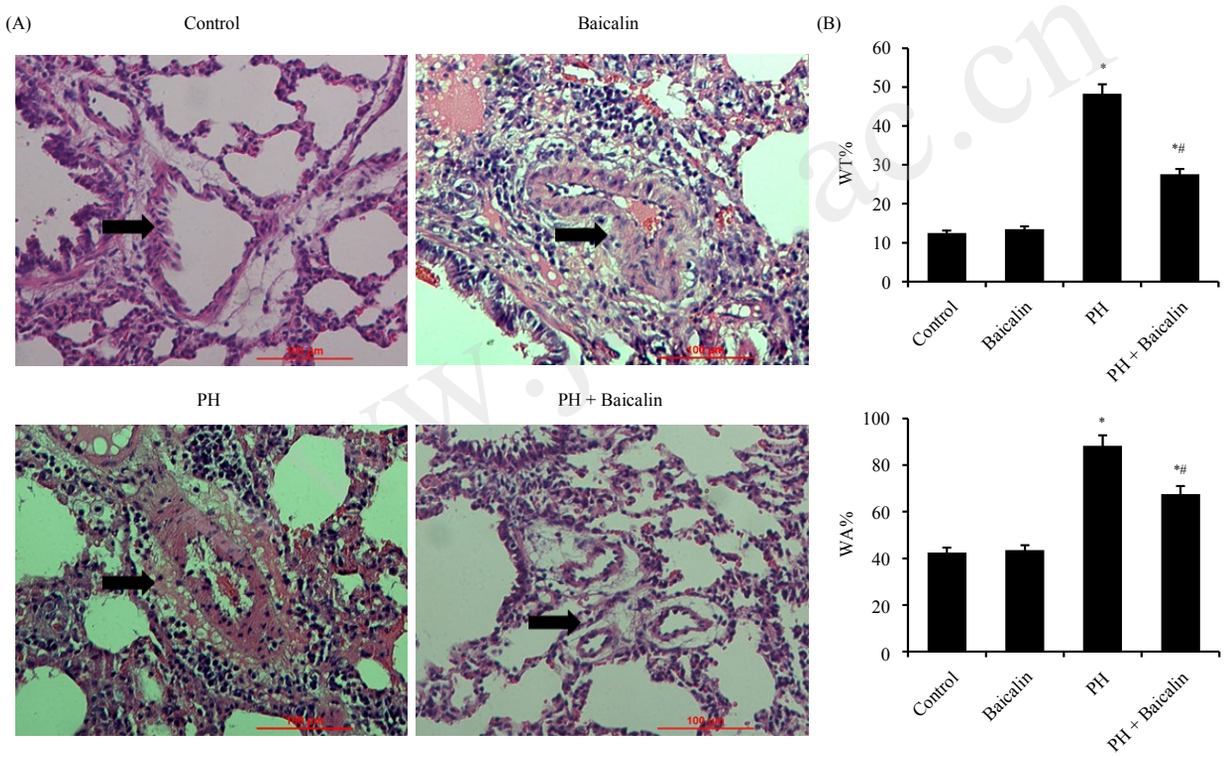

Figure 2. Effect of baicalin on MCT-induced pulmonary vascular remodeling by hematoxylin-eosin (H&E) staining. (A) Representative H&E staining images in each group. (B) A comparison of the WT% and WA% in each group. WT: vascular wall thickness, WA: vasular wall area. $^{*}P < 0.05$, *vs.* control; $^{\#}P < 0.05$, *vs.* PH group.

the lungs, and the results showed that the density was significantly decreased in the MCT group compared with the controls ($P < 0.05$). More importantly, it was significantly increased in the baicalin group as compared with the MCT group ($P < 0.05$). RT-PCR exhibited a marked reduction in the pulmonary arterial endothelial cell markers CD31 and vascular endothelial cadherin (E-cadherin). However, the mesenchymal marker α-smooth muscle actin (α-SMA) was significantly up-regulated in MCT-induced PAH rats (Fig. 3).

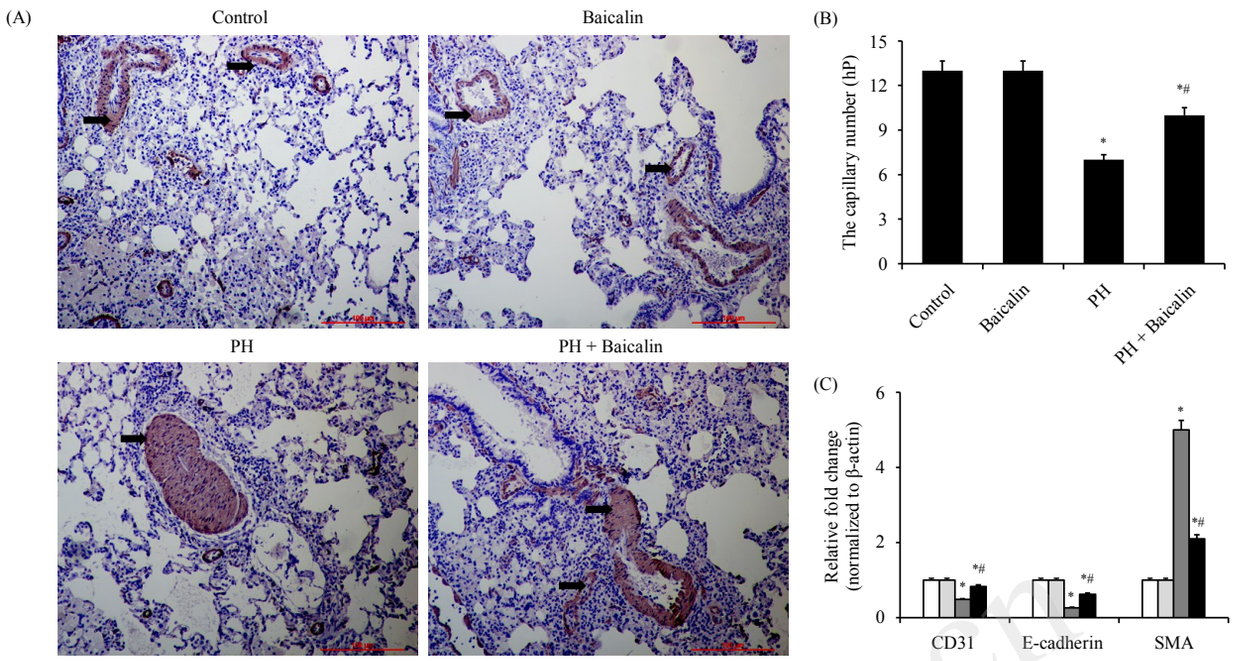

Figure 3. Effect of baicalin on pulmonary vessel density. (A) capillaries stained with CD31 in each group. (B) Comparison of the density of pulmonary arterioles in 4 groups. (C) Quantitative real-time PCR detected the mRNA expression of pulmonary arterial endothelial cell markers CD31, and vascular endothelial cadherin E-cadherin, and α-SMA. $^{*}P < 0.05$, *vs.* control; $^{\#}P < 0.05$, *vs.* PH group.

3.4. Effect of baicalin on NF-κB-BMP signaling axis molecules

To determine the alteration of cell signaling molecules contributing to the baicalin in MCT-induced PAH rats, the expressions of NF-κB-BMP signaling axis molecules were determined. Our results showed that when the rats were treated with baicalin, the protein expressions of IκB-α and BMPR2 were significantly increased, while the expressions of NF-κB p65 and p-NF-κB p65 were decreased in lung in the baicalin+PAH group compared with the PAH group ($P < 0.05$). The BMP antagonist, gremlin 1, was also measured in lungs, and there was a significant down-regulation of gremlin 1 protein in PAH + baicalin lung compared with the PAH group ($P < 0.05$, Fig. 4).

4. Discussion

Our present study demonstrated that intragastric administration of 100 mg/kg baicalin could significantly inhibit MCT-induced RVSP, RV/LV+S and PVR. The protective mechanism seemed to be associated with activation of BMP signaling, leading to attenuation of inflammatory response. Our study provided the following evidence in regards to the mechanism of baicalin inhibition on MCT-induced PAH. PAH is a severe clinical condition associated with a poor prognosis and high mortality, characterized by narrowing and obliteration of precapillary pulmonary arteries, secondary to proliferation and apoptosis resistance of endothelial cells, smooth muscle cells, and fibroblasts[11,19,20]. So far, no effective therapy is available for PAH. Recent studies have demonstrated that pulmonary vascular structural remodeling of the distal pulmonary vasculature is considered to be the major pathological basis of hypoxic PAH. However, the exact mechanism of remodeling has not yet been fully elucidated. NF-κB is a key transcriptional regulator factor, and it plays a key role in the process of vascular remodeling in a variety of physiological and pathophysiological states[21,22]. Inhibition of NF-κB in the lungs can reduce the endothelial damage,

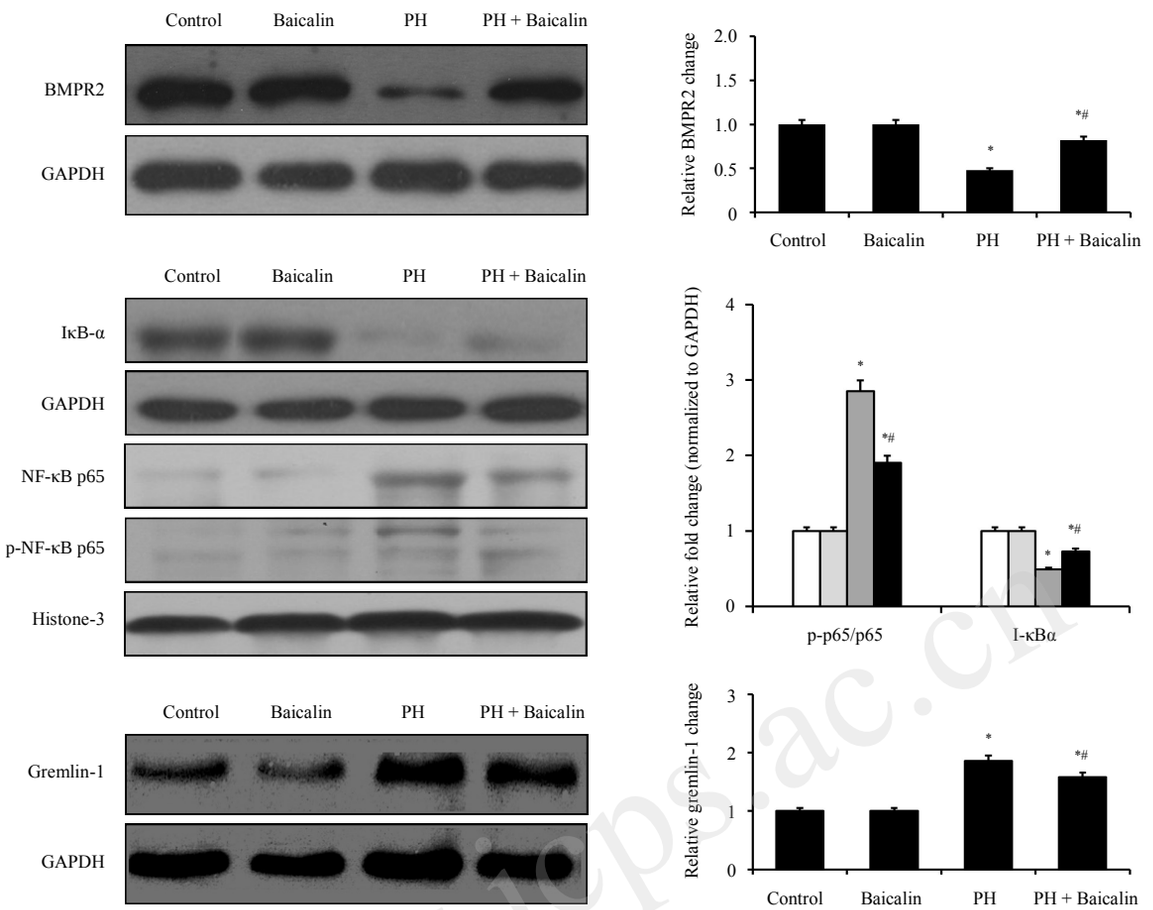

Figure 4. Analysis of protein level in the lung tissue by Western blotting. Western blots showing protein expression and comparison of BMPR2, NF-κB p65, p-NF-κB p65, I-κBα and gremlin 1. $^{*}P < 0.05$ *vs.* control; $^{\#}P < 0.05$ *vs.* PH group.

attenuate the expression of infiltrating molecules, and restore the RV pressure. Previously, we have demonstrated that baicalin can protect lung damage caused by MCT, inhibit the pulmonary artery pressure, reduce the right ventricular hypertrophy, and attenuate the PVR, and the mechanism is through inhibition of NF-κB signaling pathway[23]. However, the further signaling mechanism is unclear.

BMPR2 mutations have been reported in more than 70% of heritable cases of PAH and approximately 20% of apparently sporadic cases of idiopathic PAH[24,25]. Loss of BMPR 2 or dysfunction of BMP signaling is associated with the occurrence of PAH[11]. BMPs involve in a wide range of cell functions, including proliferation, migration, differentiation, and apoptosis. Mounting evidence indicates that several BMPs, including BMPR2, BMP4 and BMP9, play as an important role during endocrine regulator of pulmonary arterial remodeling, as well as cardiovascular, metabolic, and haematopoietic functions[8–11]. BMPR2 signaling, as a cause of increased proliferation of PASMCs, plays an important role in the remodeling of pulmonary resistance vessels in PAH[25], the expression of BMP4 is decreased in PASMCs from PAH patients compared with the controls, and BMP9 can protect pulmonary arterial endothelial cells from apoptosis, promote the vascular stability, increase the BMPR2 gene expression, further leading to enhanced BMPR2 signaling.

The induction of inhibitor of DNA binding protein (Id) expression by BMP contributes to its pro-angiogenic response. Regulation of Id proteins by BMPs, with relevance to PAH, plays a main effect in the smooth muscle cell function[24]. Id family of transcription factors, especially Id1 and Id3 as important functional targets of BMP signaling, is potently regulated by BMP signaling in PAMSCs and may play a complementary and partially redundant role in regulating cell cycling in vascular and other tissues[26,27]. Reports have previously shown a direct association of NF-κB with BMP signaling in the lungs of MCT rats, and inhibition of NF-κB attenuates PAH and RVH by regulating BMPR2–Id axis gene in heart[13]. In this report, we further confirmed that NF-κB signaling was down-regulated in the PH + baicalin group compared with the PAH group. Taken together, our data indicated the association of NF-κB-BMP signaling in the protective effect of baicalin on MCT-induced PAH and PVR.

BMP antagonists, such as gremlin and noggin, are potentially important mediators of vascular changes in hypoxic PAH, and they have been implicated in the pathophysiology of PAH[28,29]. our data showed that baicalin significantly reduced the protein expression of gremlin 1. The data provided strong evidence for the association of BMPs with the inhibition of baicalin on MCT-induced PAH, while the mechanistic link between BMP signaling pathway and PVR remains unclear and warrants future investigation.

In order to further investigation the mechanism of baicalin on BMP signaling, the Endo-MT process was also evaluated. As a developmental process, Endo-MT is characterized by the acquisition of mesenchymal phenotype, such as α-SMA, and loss of their surface marker proteins, such as CD31 and vascular endothelial cadherin. Endo-MT has also been investigated for its potential role in vascular remodeling and the fibrotic lung disease[30–32]. Recently studies have shown that Endo-MT is associated with reduced BMPR2 expression, and dysregulation of BMPR2 signaling may initiate pulmonary endothelial cell apoptosis and Endo-MT. In this study, we observed a loss of vascular endothelial cadherin and CD31 in MCT-induced PAH, which was reversed in baicalin-treated rats. Taken together, our data provided the evidence that the protection of baiclin on MCT-induced PAH might suppress the Endo-MT procession, and the underlying mechanisms were also associated with regulation of BMP signaling pathways.

In conclusion, the present study showed the protective effects of baicalin against the lung damage in MCT-induced PAH rats. Even though this effect might be related to the addition of independent beneficial effects of the treatment agents, our data at least partly suggested that baicalin exerted its mechanism in PAH through activation of BMP signaling and inhibition of PVR.

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黄芩苷通过抑制血管重构对实验性肺动脉高压的治疗作用研究

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摘要: 文献报道黄芩苷对肺动脉高压与右心室肥大具有抑制作用, 但是作用机制尚不明确。最新研究显示, NF-κB与BMP信号通路在PAH中具有重要作用, NF-κB-BMP信号轴已经成为研究肺动脉高压血管重构发病机制的重要靶点。本研究目的是观察黄芩苷对实验性肺动脉高压血管重构的抑制作用以及对NF-κB-BMP信号轴的调控作用。结果显示黄芩苷能明显降低野百合诱导的肺动脉高压右心室收缩压与右心室肥厚指数, 抑制肺动脉高压血管重构($P < 0.05$)。利用Western blot进行蛋白检测, 结果显示BMPR2蛋白表达明显提高, 而NF-κB p65、p-NF-κB p65、I-κBα与BMP抑制剂gremlin 1明显降低($P < 0.05$); 免疫组织化学结果显示肺血管密度明显提高($P < 0.05$)。以上实验结果说明, 黄芩苷对肺动脉高压肺脏和心脏损伤具有抑制作用是通过调控NF-κB-BMP信号通路, 这将为防治肺动脉高压提供新的思路与方法。

关键词: 黄芩苷; 肺动脉高压; 血管重构; NF-κB; BMP