

The protective of baicalin on pulmonary hypertension vascular remodeling through regulation of TNF- α signaling pathway

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Abstract

Background: Pulmonary hypertension (PH) is a life-threatening disease, so far no effective method for it. Baicalin can attenuate pulmonary artery pressure and reduce right ventricular hypertrophy in PH, however, the potential mechanism remains unexplored. Therefore, the main aim of the present study was to investigate the protective effect and of baicalin on experimental PH vascular remodeling, and reveal the underlying mechanism.

Methods: Monocrotaline (MCT)-induced PH rats models was established, and baicalin was given by intragastric administration. Six weeks later, right ventricular systolic pressure (RVSP) and right ventricular hypertrophy index (RVHI) were recorded, lung tissue hematoxylin-eosin (H&E) staining was analysis to reveal the effect of baicalin on MCT-induced PH. In *Vitro*, we established TNF- α induced pulmonary artery smooth muscle cells (PASMCs) to detect the inhibition of baicalin on vascular remodeling. Furthermore, quantitative real-time polymerase chain reaction (qRT-PCR), western blot and immunofluorescence were used to detect the mRNA and protein expressions.

Results: Our results indicated that baicalin could significantly attenuate MCT induced the RVSP and the right ventricular hypertrophy index (RVHI); inhibit pulmonary vascular remodeling and lung fibrosis. Moreover, our results showed baicalin could significantly decrease the expression of inflammatory cytokines, but increase the protein expression of bone morphogenetic protein type II receptor (BMP2), ID1 and Smad1//5/8.

Conclusion: Taken together, our present study confirmed the mechanism of baicalin against PH was associated with inhibition of TNF- α signaling pathway.

Introduction

Pulmonary hypertension (PH) is a life-threatening disease, is characterized by excessive proliferation of pulmonary vascular cells, eventually leads to pulmonary vascular resistance, vascular remodeling, right ventricular hypertrophy and failure [1,2]. Various types of PH prevalence exceed 30–50/1 million every year with onset around the middle-aged (75%, 20–40 years old). The pathogenesis of PH is complicated, so far, there is no effective treatment for it and the mortality rate is still high.

A large number of reports [3–5] showed that excessive proliferation of pulmonary artery smooth muscle cells (PASMCs) is an important pathogenesis in PH vascular remodeling process. Bone morphogenetic protein type II receptor (BMP2) is a key factor in the process of PH pulmonary remodeling occurs[6,7]. BMP2 levels in pulmonary vascular was significant reduced in non-genetic forms of PH. More than 70% of heritable and 20% of idiopathic PH cases have the BMP2 *gene* mutations. Recent studies have found that inflammation is thought to be strong trigger factors and promotes the development of PH in *Bmpr2*^{+/-} mice, inflammatory play an important role in PAH [7–9], inflammatory factors was significantly increased in clinical PH patient. Inflammatory mediator tumor necrosis factor- α (TNF- α) could further promote the development of PH by reducing BMP2 expression in PASMCs.

Scutellaria baicalensis Georgi (*huang qin*) is a widely used herb in traditional Chinese medicinal herbs. The study found that baicalin has several biological effects including antioxidant, anti-inflammatory, antiviral, neuroprotective, anxiolytic, and anti-cancer activities[9–13]. Our previous study showed that baicalin could inhibit inflammatory, improve PH vascular remodeling and right ventricular impairment in monocrotaline (MCT)-induced experimental[14,15]. However, the mechanism is still unclear.

In the present study, we tested the hypothesis that baicalin have the therapeutic effect on PH through regulation of TNF- α /BMP2 signaling pathway and further inhibiting pulmonary vascular remodeling. These will provide theoretical foundation for the clinical treatment of PH.

Materials And Methods

Animals experimental design

Baicalin (purity > 95%) was purchased from Sigma (St. Louis, MO, USA) and was dissolved in dimethyl sulfoxide (DMSO). The PH model was induced by intraperitoneal injection of 60 mg/kg MCT for 6 weeks (Sigma-Aldrich, USA) as our previously described with modifications[15]. 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 4 groups: Control, MCT, saline and baicalin treated groups (n = 10 in each).

Hemodynamic and the right ventricular hypertrophy assessment

All animals were anaesthetized by isoflurane inhalation (1.5-2%) and then euthanized by cervical dislocation. Hemodynamic data were recorded after operation as previously described with some modifications. Via femoral vein access, a 5F Swan-Ganz catheter (Edwards Lifesciences Corp, Irvine, CA) 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) plus the septum (LV + S) were harvested, and the weight ratio of the RV to LV + S weight calculated to quantify the right ventricular hypertrophy. The right ventricular hypertrophy index (RVHI) by the formula: $RV/(LV + S) \times 100$.

Immunological and immunohistochemical analyses

Post-operation, the lung and heart were quickly harvested and fixed in 4% paraformaldehyde and embedded in paraffin, the serially sectioned at a thickness of 4–5 μ m were stained with hematoxylin-eosin (H&E). To evaluate pulmonary artery structural remodeling, the vascular wall thickness (WT), vascular external diameter (ED), vascular wall area (WA) and total vascular area (TA) to calculate WT% (WT/ED) and WA% (WA/TA) were measured as previously study. Fibrosis area analysis 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 were then carried out to analysis the expression of smooth muscle actin (α -SMA) by immunohistochemistry and immunofluorescence. Briefly, after blocking unspcific protein binding with 5% bovine serum albumin for 30 min at room temperature, the lung sections were incubated overnight at 4 °C with antibodies. Images were taken with an Eclipse 90i microscope (Nikon, Tokyo, Japan). Staining was quantified using Image Pro Plus 6.0 image analysis software (Media Cybernetics, Rockville, MD). All experiments were performed by two examiners blinded to treatment assignment.

Cell experiment

Rats PAMSCs were purchased from Procell Life Science&Technology Co.,Ltd. (Wuhan, China), and cultured in special culture medium (Procell, China) supplemented with 100 Ug/ml of penicillin, 100 IU/ml streptomycin, and 10% (vol/vol) fetal bovine serum (FBS) at 37 °C. Cells were pass-aged after > 80% confluence, digested with 0.05% trypsin including 0.04% EDTA (Sigma-Aldrich, St. Louis, MO) in PBS. Recombinant rat TNF- α was purchased from PeproTech (Cat:400 - 14, Rocky Hill, NJ). Cells were treated with TNF- α (5 ng/ml) for 24 h at 37 °C in the presence or absence of baicalin (100 μ g/ml), respectively.

Cells proliferation and migration assay

PASMCs 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 ($1-5 \times 10^4$ cells/well), after confluence > 80%, 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 incubated with MTT (5 mg/mL) for 4 h, absorbance was measured at 490 nm.

The migratory function of PAMSC was evaluated using a modified Boyden chamber (Transwell; Corning Life Sciences, Inc., Tewksbury, MA, USA) assay with a polycarbonate filter with 8- μ m pores placed between the upper and lower chambers. In brief, at 0 and 24 h following TNF- α pre-treatment, 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 a 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., Beijing, China). The number of migrated cells was counted using a Nikon Eclipse 90i microscope.

RNA preparation and quantitative reverse transcription-PCR

Total RNA was extracted using the RNeasy Mini Kit with DNase digestion (Qiagen, West Sussex, UK) from lung tissues and cultured PAMSCs. Quantitative real-time polymerase chain reaction(qRT-PCR) analysis was performed using a M \times 3000P System. 1.5% agarose gel electrophoresis in the presence of ethidium bromide (Sigma-Aldrich) was used to amplification fragments, and β -actin as and internal control. Primers were designed using the Primer Express software package (Applied Biosystems, Foster City, CA, USA): TNF- α : 5'-AAATGGGCTCCCTCTATCAGTTC-3'(forward primer) and 5'-TCTGCT TGGTGGTTTGCTACGAC-3'(reverse primer); IL-1 β , 5'-CCTTGTGCAAGTGTCT GAAGC-3'(forward primer)

and 5'-CCCAAGTCAAGGGCTTGGAA-3(reverse primer);IL-6:5'-AAGTCGGAGGCTTAATTACACATGT-3', (forward primer) and 5'-AAGTGCATCATCGTTGTTTCATACA-3'(reverse primer); β -actin: 5'-TCTACA ATGAGCTGCGTGTG-3'(forward primer) and 5'-GGTCAGGATCTTCATGA GGT-3'(reverse primer). The relative gene expression level was determined using the $2^{-\Delta\Delta CT}$ method.

Western blot analysis

The tissues and cells protein concentration was detected using a BCA assay kit, lysates were separated by polyacrylamide gel electrophoresis (PAGE) and electro-transferred onto a polyvinylidene fluoride (PVDF), the membranes 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, Billerica, Ma, USA) followed by Bio-Rad Image Lab™. Data was expressed as the relative density of the protein normalized to GAPDH. Primary antibodies of α -SMA (ab21027), BMPR2 (ab170206), NF- κ B-p65 (ab16502), p-NF- κ B-p65 (ab86299), Smad1/5/8 (sc-6031), p-Smad1/5/8 (sc-12353), ID1 (ab168256), Cyclin D1(MA5-15512), P27^{Kip1}(ab32034), VCAM-1(ab134047) and ICAM (ab171123) were used, respectively.

Ethics

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 anaesthetized by isoflurane inhalation (1.5-2%) and then euthanized by cervical dislocation. patients. The work was done in accordance with the Helsinki Declaration's guidelines.

Statistical analysis

All data are expressed as mean \pm SD. Comparisons of parameters between 2 groups were made with unpaired Student t test. Comparisons of parameters among 3 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. $P < 0.05$ was regarded as significant statistical difference.

Results

Effect of baicalin on PH vascular remodeling

Six weeks later, we evaluated MCT-induced lung and heart injury by detecting RVSP and right ventricular hypertrophy index $RV/(LV + S)$, as shown in Fig. 1A, a significant increased of RVSP and $RV/(LV + S)$ in MCT administration group as compared with control ($P < 0.05$), these indicated that we successfully established PH model in rats. However, RVSP and $RV/(LV + S)$ were significantly inhibited in baicalin treated rats than that in MCT rats ($P < 0.05$). There was no significantly different in HR and SBP between groups ($P > 0.05$).

Lung sections were stained with H&E and Masson's trichrome was used to analysis the medal thickness of pulmonary arterial walls and the degree of fibrosis. As shown in Fig. 1B and 1C, 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 ($P < 0.05$). Masson's stained results showed that the lung fibrosis was significantly reduced in baicalin treated group than that in MCT group (Fig. 2A and 2B, $P < 0.05$).

Effect of baicalin on smooth muscle cells

In this study, smooth muscularization cells marker α -SMA were analyzed by immunohistochemistry and immunofluorescence. The results showed that the expression of α -SMA were significantly increased in MCT group than that in control, however, which were significantly lower in baicalin treated group ($P < 0.05$, Fig. 3A and 3B).

Effect of baicalin on inflammatory response

To explore the underlying mechanisms of baicalin against pulmonary vascular remodeling, the mRNA levels of interleukin (IL)-1 β and IL-6 were analyzed by qRT-PCR, the proteins expression of TNF- α , VCAM-1 and ICAM were detected by western blot. The results showed that the inflammatory factors TNF- α , VCAM-1, ICAM and the ratio of phosphorylated to total NF- κ B-p65 levels were obviously increased in lung tissue when the animals were subjected to MCT, but which were significantly reduced in baicalin treated group rats as compared with MCT rats ($P < 0.05$, Fig. 4A-4C).

Effect of baicalin on BMPR2 signaling pathway in vivo

BMPR2 signaling playing an important role in the remodeling in PH, in the present study, we detected the protein expression of BMPR2, Smad1/5/8, p-Smsd1/5/8 and ID1 by western blot. Our results showed that an obviously up-regulation of protein expression of BMPR2, Smad 1/5/8, p-Smsd1/5/8 and ID1 in baicalin treated group than those in MCT groups ($P < 0.05$, Fig. 5).

Effect of baicalin on PAMSC proliferation and migration

The effect of baicalin on proliferation and migration abilities of PAMSC were analyzed in *vitro*. Briefly, after cells were treated with TNF- α for 48 h and following treated with 100 $\mu\text{g}/\text{ml}$ baicalin for 24 h, 48 h and 72 h. MTT assay results exhibited that the cells viability rate was significantly increased in TNF- α group as compared with the normal groups. However, when the cells were pretreatment with baicalin, which was significantly inhibited ($P < 0.05$, Fig. 6A).

Transwell assay was performed to observe the effect of baicalin on cells migratory ability. As demonstrated in Fig. 5B, the migratory ability was significantly enhanced when the cells were induced by TNF- α for 48 h, but which was obviously inhibited by baicalin ($P < 0.05$, Fig. 6B and 6C).

Furthermore, western blot results showed that the protein expression of Cyclin D1 was significantly increased, but the protein expression of P27^{kip1} was significantly decreased in MCT group than that in control. However, baicalin administration could significantly restore these results than that in MCT rats ($P < 0.05$, Fig. 6D).

Effect of baicalin on TNF- α induces BMPR2 signaling

To further explore the underlying mechanisms, the proteins expression of BMPR2 signaling were analyzed by immunofluorescence and western blot in *vitro*. The results showed that the protein expression of BMPR2, ID1 and p-Smad1/5/8 were significantly suppressed in TNF- α induced group, however, which were restored when the cells were treated with baicalin ($P < 0.05$, Fig. 7).

Discussion

Pulmonary hypertension (PH) is a kind of refractory rare lung diseases, distal pulmonary arterial remodeling is the characteristic of it [16–18]. The pathogenesis of PH is not clear yet, and no effective therapy is available for it. Previous studies [14,15] suggested the baicalin has the potential to inhibition of vascular remodeling in PH. In the present study, our data confirmed that administration of baicalin could significantly reduce RVSP and RV/(LV + S) as compared with the MCT rats, which is considered as a novel potential therapeutic approach for PH.

Inflammatory was associated with the pathogenesis of PH [9,19], which play an main role in initiating and maintaining vascular remodeling in PH animal models, and therapeutics targeting of inflammation maybe attenuate the development of PH [20]. Reports showed that TNF- α levels was significantly higher in heritable and idiopathic PH cases as compared with healthy people [9,21,22]. The present study showed significantly decreased the mRNA and protein levels of TNF- α , IL-1 β , IL-6 and NF- κ B in baicalin group than that in MCT-induced PH group. These results indicate that baicalin could suppress the inflammatory response in MCT-PH rats. We therefore analysis the protein expressions of the adhesion molecules VCAM-1, ICAM and ratio of phosphorylated to total NF- κ B-p65 levels which were also involved in inflammatory processes [23,24]. Collectively, these results showed that baicalin attenuated MCT-induced inflammation as evidenced by decreased expressions of adhesion molecules.

Bone morphogenetic proteins (BMPs) and their receptors were required for PH-induced right ventricular hypertrophy, which playing an important role in the remodeling of pulmonary resistance vessels in the process of PH occurs [8,25,26]. BMPR2 was significantly decreased in MCT and chronic hypoxic induced rat PH models [27,28]. BMP signaling through receptor-mediated phosphorylation and p-Smad1/5/8 transcription factors and alterations in gene transcription to regulation the occurrence of pulmonary fibrosis process. In addition to BMPR2, BMP2, BMP4, BMP6 and BMP9 also have regulatory role for pulmonary vascular cell proliferation [29–32]. In the present study, we found that the protein expression of BMPR2, Smad1/5/8, p-Smad1/5/8 and ID1 were significantly up-regulation by baicalin. Taken-together, these results confirmed that baicalin could significantly repair of MCT-induced PH pulmonary vascular remodeling through regulation of the BMP signaling pathways.

Recent study showed that the expression of BMPR2 was inhibited by TNF- α in pulmonary vascular cells [33] and pulmonary artery endothelial cells [34]. Many cytokines involved in the pathogenesis of PH, such as TNF- α , IL-1 β , IL-6 and IL-8 are implicated in the pathogenesis of PH, only TNF- α selectively reduced BMPR2 expression in distal PSMCs and PAECs. On the other hand, TNF- α could promote

pulmonary vascular remodeling in the setting of BMPR2 deficiency, increase BMP6 expression caused up-regulation in transient p-Smad1/5/8 responses in PASMCs[35].

To further explore the underlying mechanism of baicalin for PH. We used TNF- α induced PAMSC injury *in vitro* in the presence or absence of baicalin. The effect of baicalin on proliferation and migration abilities of PAMSC were also analyzed, the MTT and Transwell results showed that the viability and proliferation rate were significantly increased after cells were treated with TNF- α as compared with the normal cells. However, baicalin administration could significantly inhibit the cells proliferation and migration. Furthermore, TNF- α induce PAMSC proliferation was also observed by detecting the protein expression of Cyclin D1 and P27, the results showed that the expression of CyclinD1 was significantly higher, but P27 was significantly lower in baicalin group than that in TNF- α group. More-importantly, in the present study, our results showed that the proteins expression of BMPR2 signaling were significantly up-regulation in baicalin administration group than that in TNF- α group. Therefore, our data provide a strong evidence for the mechanism of baicalin inhibition PH vascular remodeling was to through down-regulation TNF- α drives PH by suppressing the BMPR2 signaling.

In summary, the present study demonstrated for the first time that baicalin attenuated PH pulmonary vascular remodeling through inhibition of inflammatory response, but increased the vascular remodeling associated BMPR2 signaling pathway.

Declarations

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Availability of supporting data

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

Ethical Approval and Consent to participate

All participants were provided with written informed consent at the time of recruitment. And this study was approved by the Ethics Committee of the Second Hospital of Shanodng University.

Consent for publication

Not applicable.

Author Contributions

YL designed the research and oversaw the writing of the manuscript; XX, JW, WJ,QX, KLL and TGQ performed the experiments and wrote the manuscript, YL and JW analyzed the data. All authors have read and approved the manuscript.

Competing interests

The authors declare that they have no competing interests.

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Figures

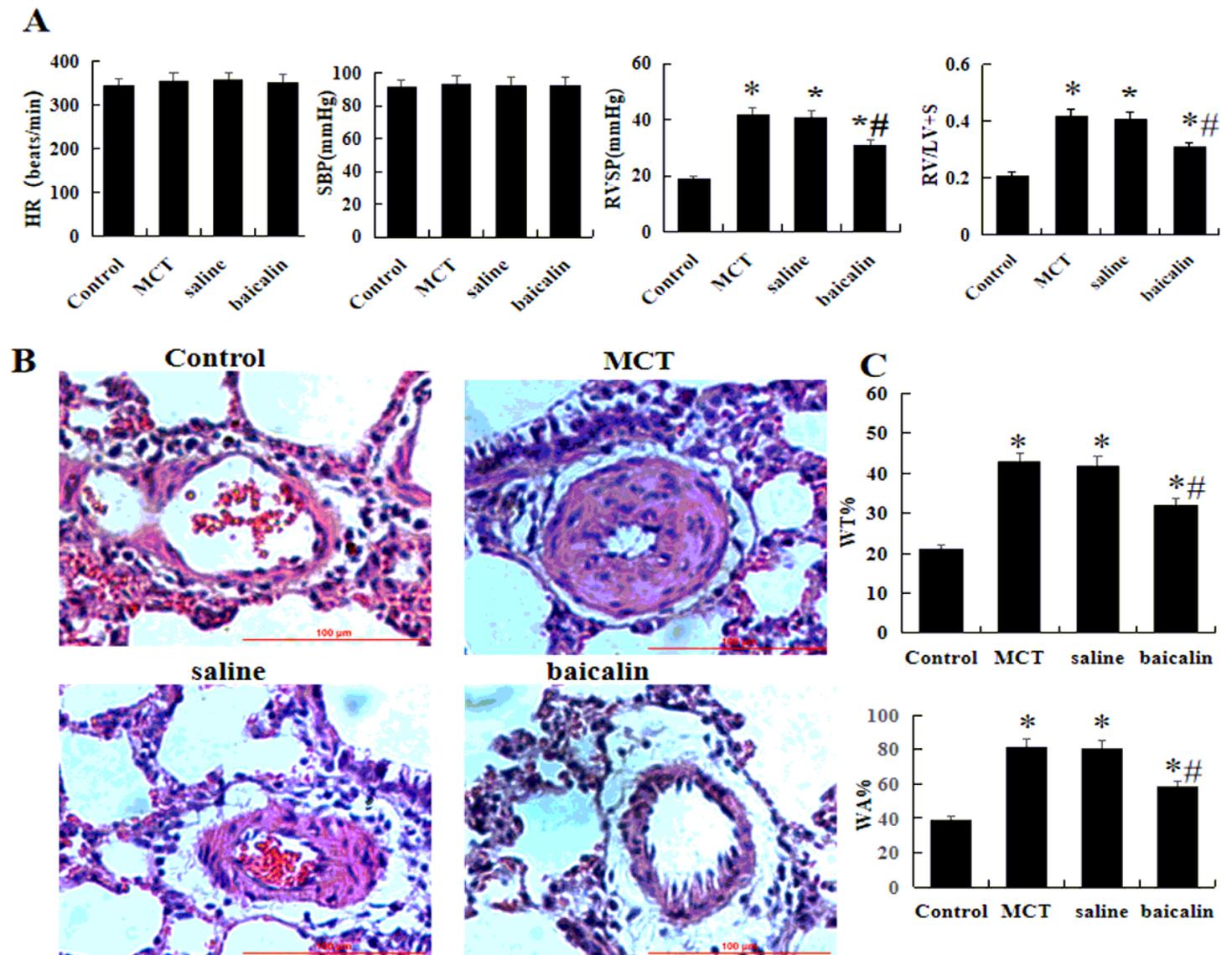


Figure 1

Effect of baicalin on MCT-induced pulmonary hypertension (A) 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. MCT: monocrotaline; HR: heart rate; SBP: systemic blood pressure; RVSP: right ventricular systolic pressure; RV/LV+S: the ratio of right ventricular weight to left ventricle plus septum; WT%: the percent of the vascular wall thickness (WT)/vascular external diameter (ED); WA%: the percent of vascular wall area (WA)/total vascular area (TA); the data are present as mean \pm SD; * P <0.05 and ** P <0.01 compared with control group; # P <0.05 compared with MCT or saline.

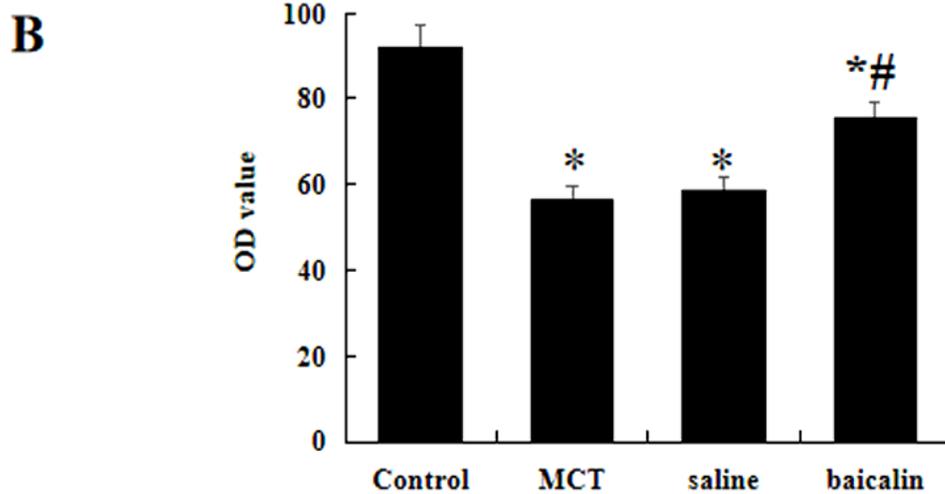
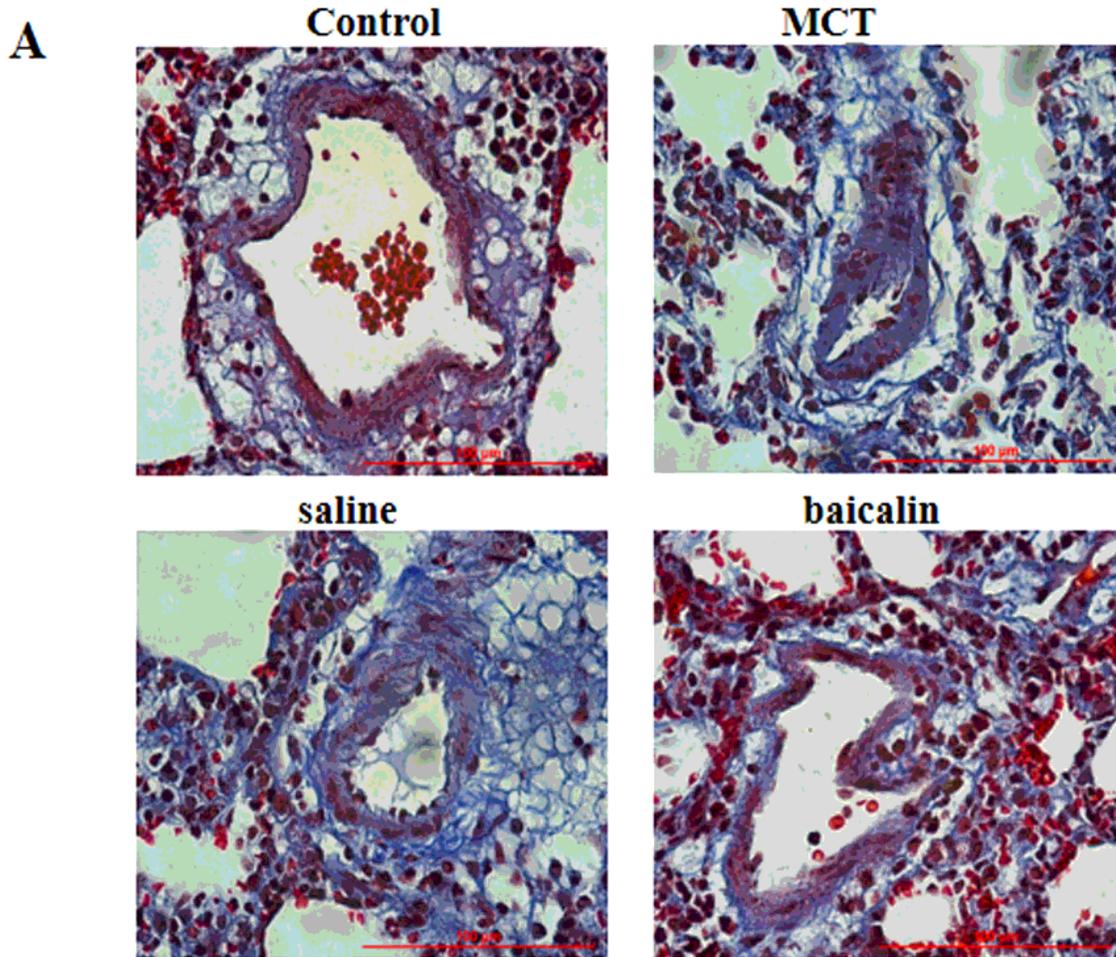


Figure 2

Effect of baicalin on MCT-induced lung fibrosis (A) Massons staining. (B) A comparison of the OD value in each group. OD: optical density calibration. the data are present as mean \pm SD; * $P < 0.05$ and ** $P < 0.01$ compared with control group; # $P < 0.05$ compared with MCT or saline

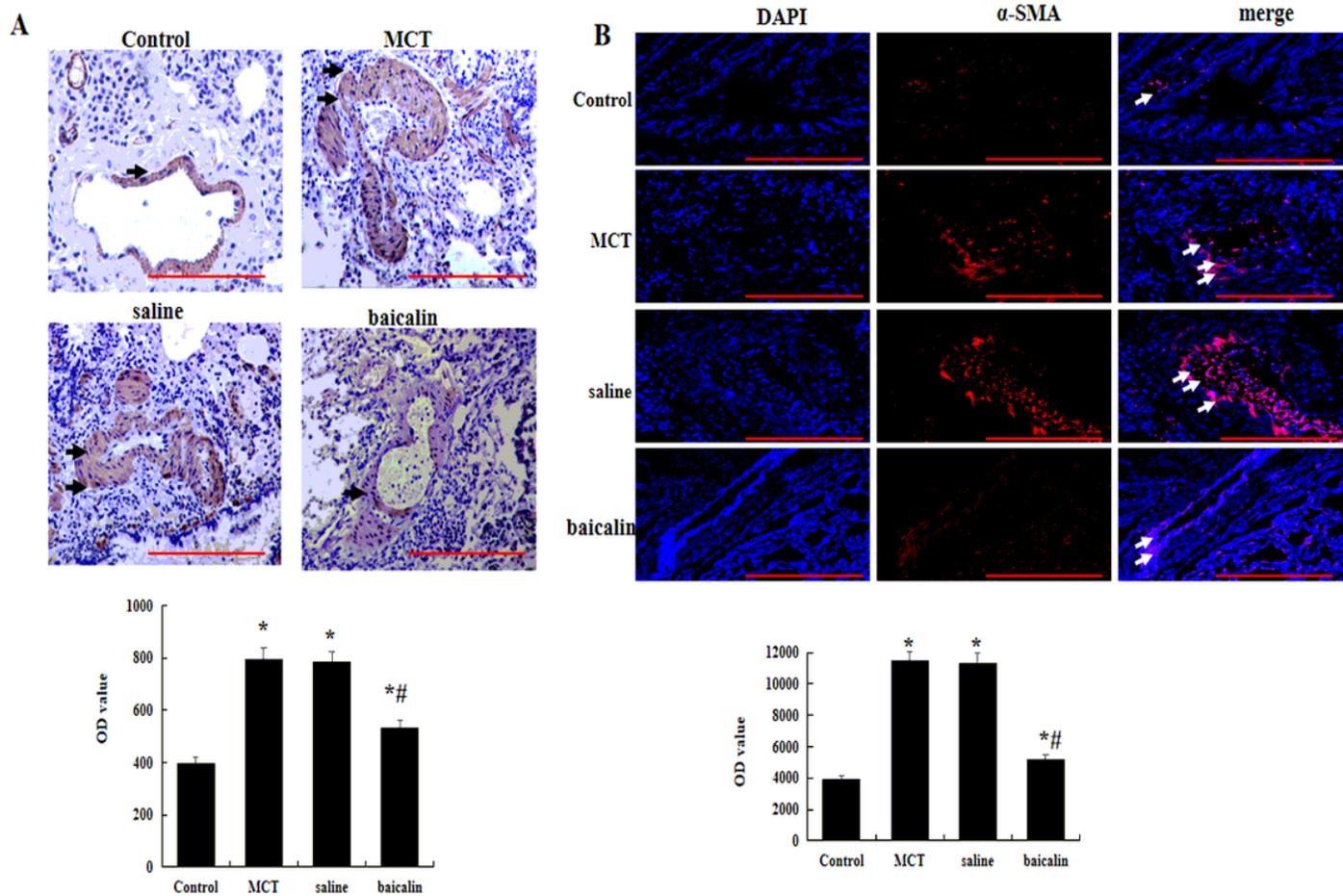


Figure 3

Effects of baicalin on MCT-induced pulmonary artery smooth muscle (A) The expression of α -smooth muscle actin (α -SMA) analysis by immunohistochemical. (B) The expression of α -SMA analysis by immunofluorescence. OD: optical density calibration. t the data are present as mean \pm SD; * $P < 0.05$ compared with control group; # $P < 0.05$ compared MCT or saline group.

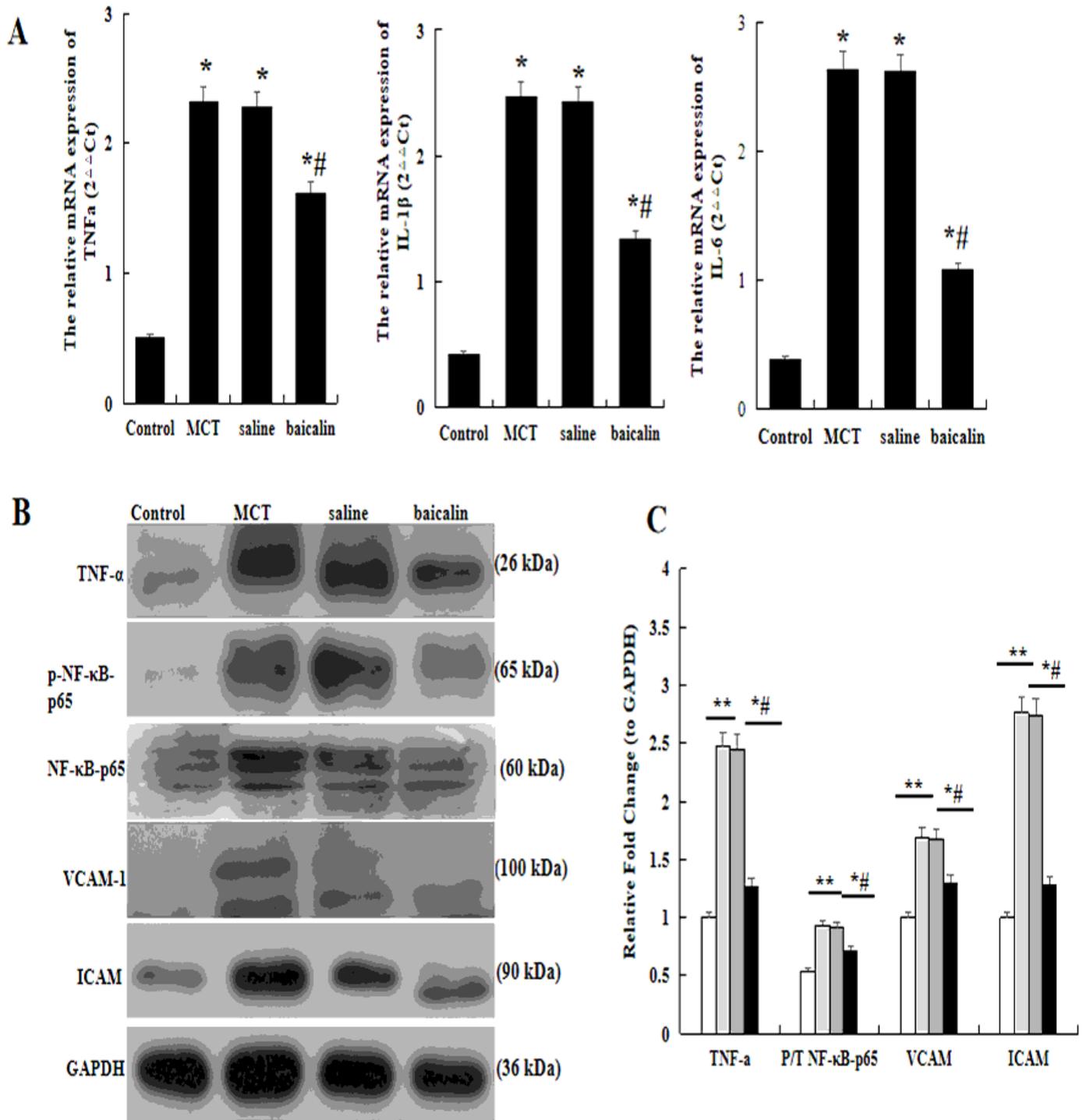


Figure 4

Effect of baicalin on inflammatory in the lung (A) Quantitative real-time polymerase chain reaction analysis the mRNA level of tumor necrosis factor- α (TNF- α), interleukin (IL)-1 β and IL-6. (B) Western blots analysis the protein expression of TNF- α , Phosphorylated Nuclear factor- κ B-p65 (p-NF- κ B-p65), Total NF- κ B-p65, intercellular cell adhesion molecule-1 (ICAM1) and vascular cell adhesion molecule(VCAM). (C) Normalized band intensity quantification showing the fold change of the TNF- α , the ratio of P to Total

NF- κ B-p65 (P/TNF- κ B-p65), VCAM-1 and ICAM in each group. the data are present as mean \pm SD; *P<0.05 compared with control group;#P<0.05 compared with MCT or saline group.

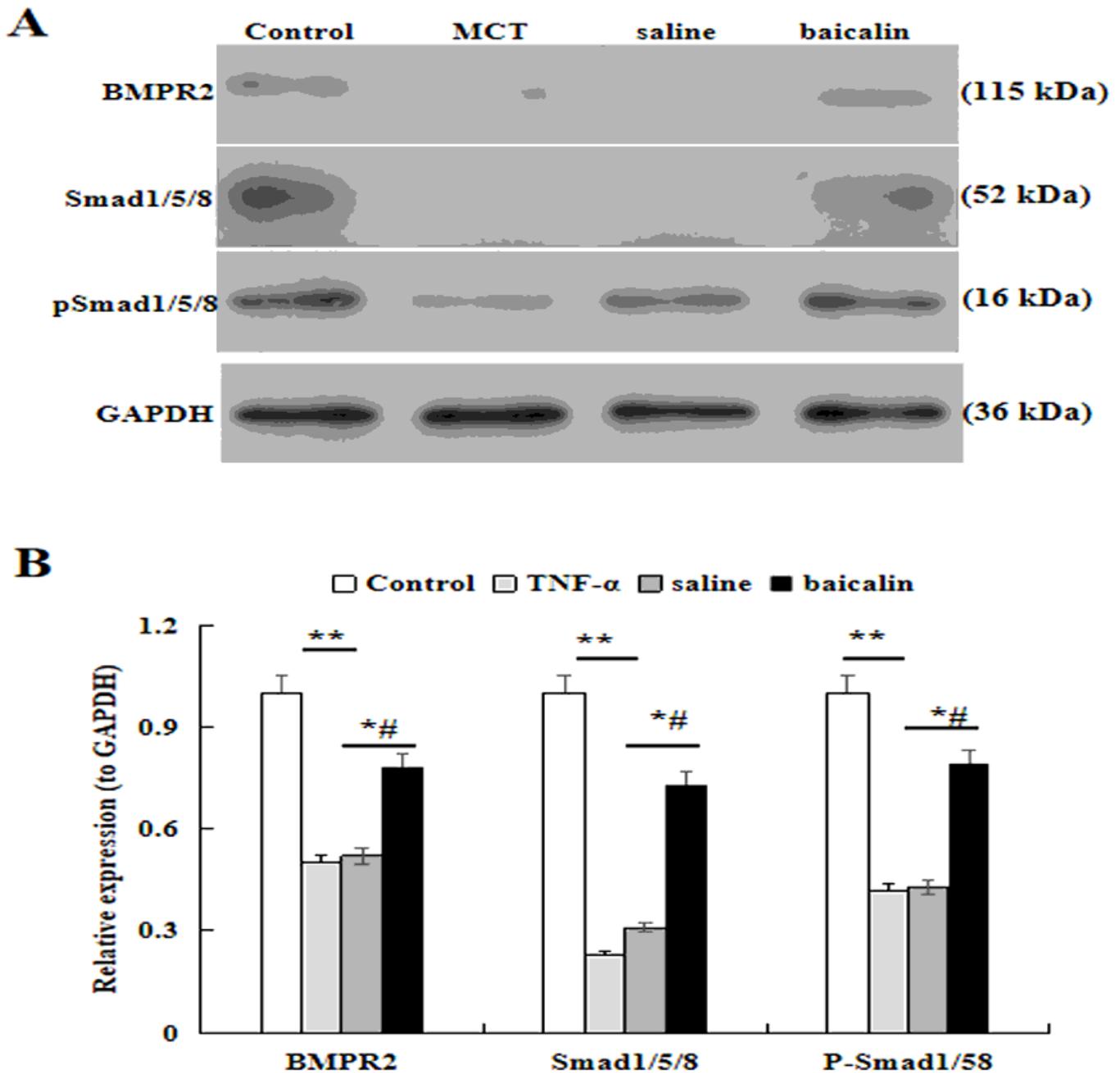


Figure 5

Effect of baicalin on BMPR2 signaling pathway in the lung (A) Western blots analysis the protein expression of bone morphogenetic protein type II receptor (BMPR2), p-Smad1/5/8 and ID1 in lung tissue. (B) Normalized band intensity quantification showing the fold change of BMPR2, Smad1/5/8, p-

Smad1/5/8 and ID. the data are present as mean \pm SD; *P<0.05 compared with control group;#P<0.05 compared with MCT or saline group.

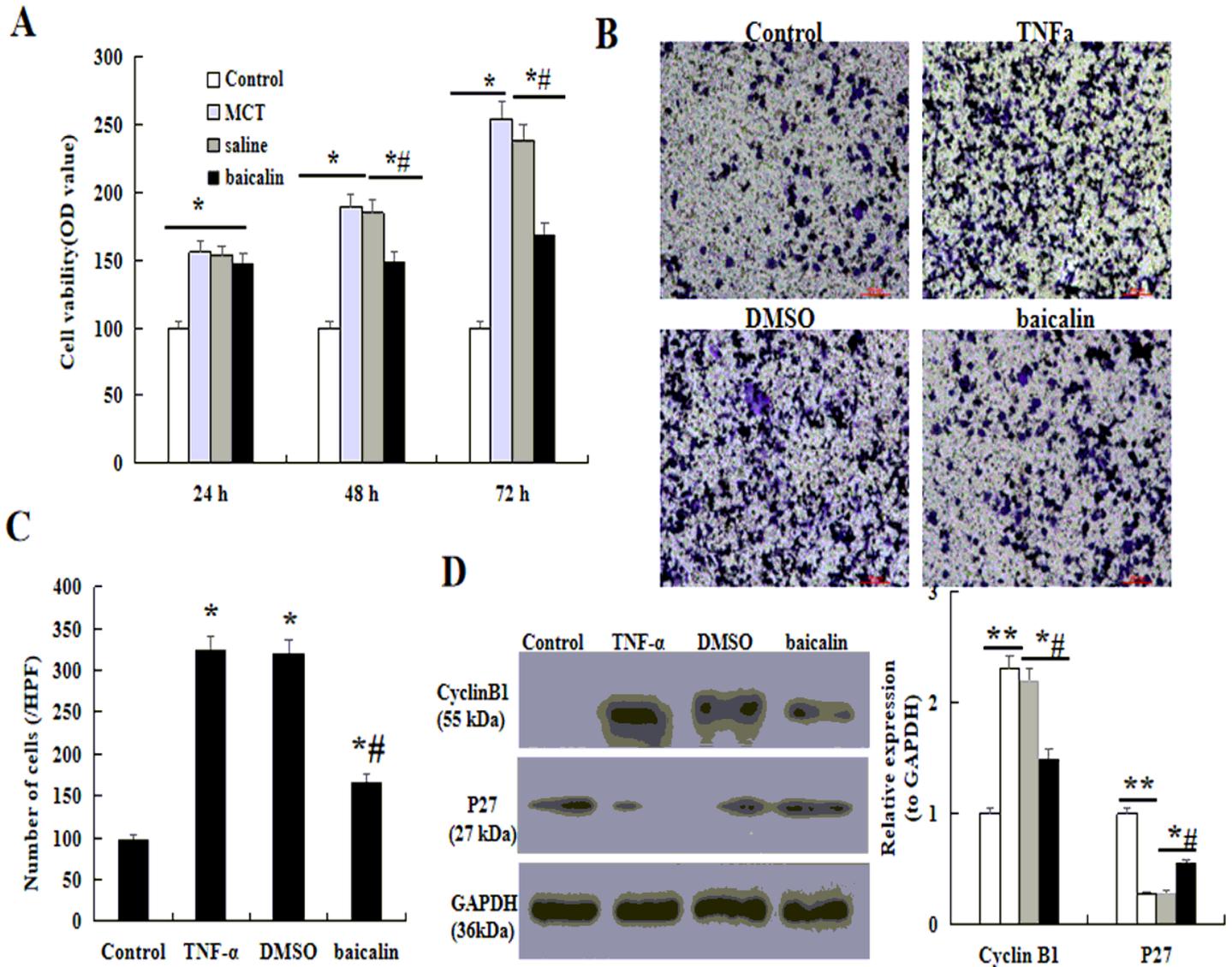


Figure 6

Effect of baicalin on TNF- α -induced PAMSC (A) MTT assay. (B) Transwell assay. (C) Comparison of number of cells. (D) the protein expression of Cyclin D1 and p27 analysis by western blot. the data are present as mean \pm SD; *P<0.05 compared with control group;#P<0.05 compared with MCT or saline group.

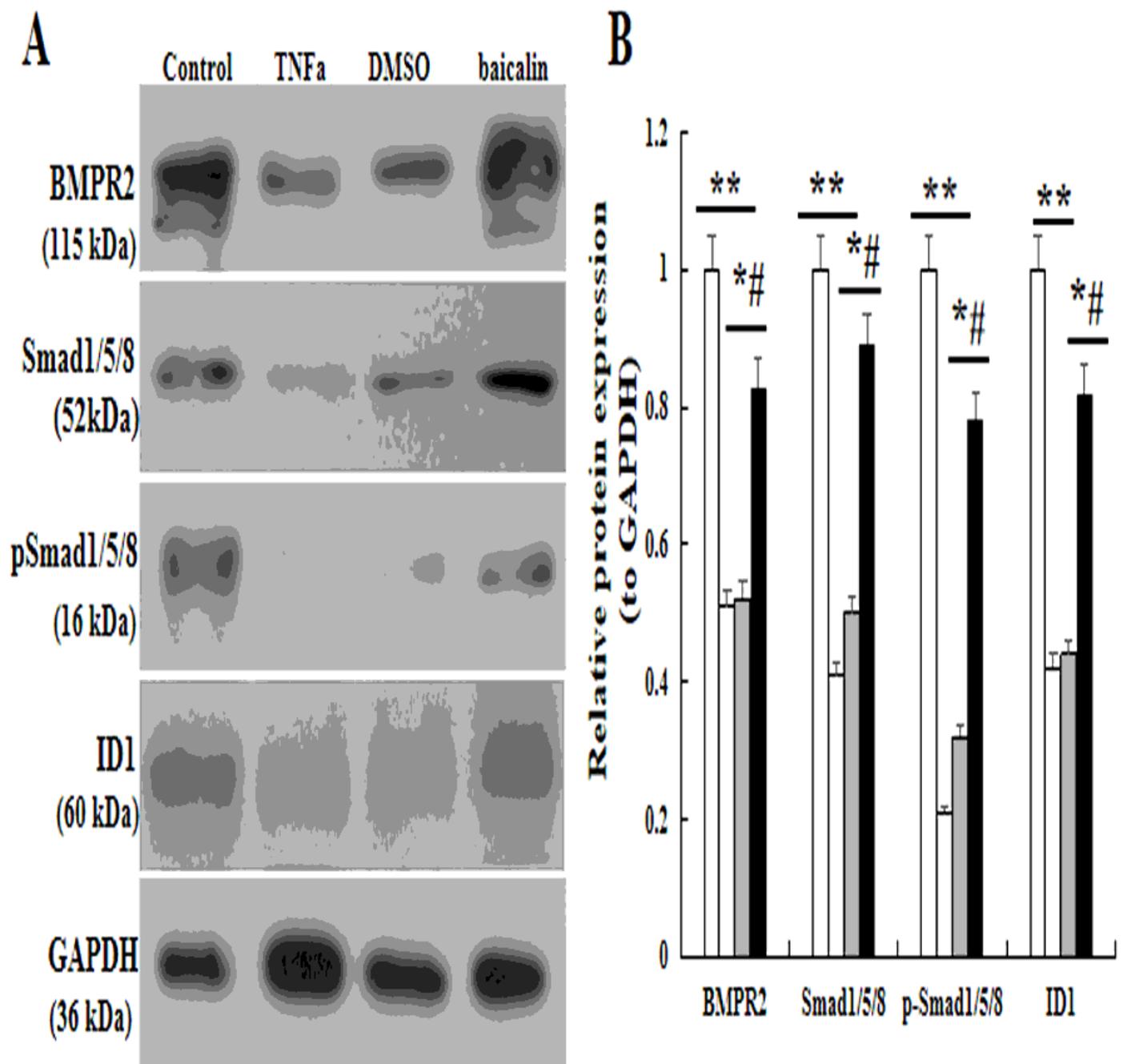


Figure 7

Effect of baicalin on BMPR2 signaling pathway in vitro (A) the protein expression of BMPR2, Smad1/5/8, p-Smad1/5/8 and ID1 analysis by western blot. (B) Normalized band intensity quantification showing the fold change of BMPR2, Smad1/5/8, p-Smad1/5/8 and ID. the data are present as mean \pm SD; * $P < 0.05$ compared with control group; # $P < 0.05$ compared with TNF- α .