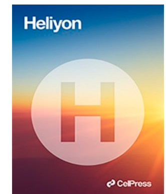
Heliyon 9 (2023) e23076

Contents lists available at ScienceDirect

Heliyon

journal homepage: www.cell.com/heliyon



Baicalin attenuates pulmonary vascular remodeling by inhibiting calpain-1 mediated endothelial-to-mesenchymal transition

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ARTICLE INFO

Keywords:
Baicalin
Pulmonary vascular remodeling
Endothelial-to-mesenchymal transition
Calpain-1
PI3K/Akt
Pulmonary arterial hypertension

ABSTRACT

Background: Previous studies have demonstrated the beneficial effect of baicalin on pulmonary arterial hypertension (PAH), but the mechanism is unclear.
Aim: The aim of the present study was to evaluate the effect of baicalin on pulmonary vascular remodeling (PVR) with a focus on calpain-1-mediated endothelial-to-mesenchymal transition (EndMT).
Methods: PAH was induced by intraperitoneal injection of monocrotaline (MCT) in rats and hypoxia in calpain-1 gene knockout (Capn1$^{-/-}$) and wild-type C57BL/6 mice. An in vitro PVR model was established in PASMCs and HPAECs.
Results: The data showed that baicalin treatment and calpain-1 inhibition alleviated MCT and hypoxia-induced increases in right ventricular systolic pressure (RVSP), prevented right ventricle hypertrophy and PVR, and attenuated cardiopulmonary fibrosis. Moreover, baicalin ameliorated PAH-induced EndMT, as evidenced by the suppressed expression of mesenchymal markers vimentin, and α-SMA and restored expression of endothelial markers CD31, and VE-cadherin. In vitro studies showed that baicalin treatment blocked TGF-β1-induced EndMT in HPAECs and abolished hypoxia-induced PASMC proliferation and migration. All the beneficial effects of baicalin on PVR in vitro and in vivo were accompanied by suppressed calpain-1 expression. Further study demonstrated that baicalin treatment and calpain-1 inhibition inhibited the enhanced expression of PI3K and *p*-AKT both in vitro and in vivo.
Conclusions: In conclusion, baicalin treatment attenuates PVR by inhibiting calpain-1 and PI3K/Akt-mediated EndMT.

1. Introduction

Pulmonary arterial hypertension (PAH) is a group of heterogeneous diseases with a progressive increase in pulmonary artery pressure, which leads to right-sided heart failure and that could be lethal in some cases. PAH has a high incidence and mortality and is

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https://doi.org/10.1016/j.heliyon.2023.e23076
Received 3 July 2023; Received in revised form 24 November 2023; Accepted 27 November 2023
Available online 30 November 2023
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considered as a puzzle in the cardiovascular field. The pathogenesis of PAH is multifactorial and complex, but it eventually leads to pulmonary vascular remodeling (PVR). Endothelial dysfunction and smooth muscle cell proliferation are important components of PVR [1–3]. However, accumulating evidence highlights the contribution of endothelial-to-mesenchymal transition (EndMT) to the development of PVR [4–6]. EndMT is a biological process in which endothelial cells change their endothelial phenotype into a mesenchymal or myofibroblast phenotype. Stimulated by some pathological factors, endothelial cells lose their endothelial markers (VE-cadherin and CD31), acquire markers of mesenchymal cells (α-SMA), and gain migratory and invasive capacities [7,8]. Mesenchymal endothelial cells promote the formation and progression of occlusive intimal lesions and aggravate the occurrence of PVR [9,10]. Therefore, EndMT intervention might be a novel and effective therapeutic strategy for PAH.

Calpains are calcium-dependent cysteine hydrolases that play a role by degrading related functional proteins or enzymes in the body. Calpain-1 (μ-calpain) and calpain-2 (*m*-calpain) are the two main members of the calpains, differentiated according to the concentration of calcium ions required for activation. Both calpain-1 and calpain-2 are composed of 80 kD large subunits with catalytic activity and 28 kD small subunits with regulatory activity. Calpains are regarded as molecular targets for a variety of cardiovascular diseases, including atherosclerosis, hypertension and diabetes [11–13]. Studies have shown that calpain mediates myocardial endothelial cell (EC)-mesenchymal transformation through the HSP90/protein kinase B (AKT) pathway, participates in myocardial endothelial injury, and cell proliferation ultimately leads to myocardial fibrosis [14]. Overexpression of calpastatin, an endogenous inhibitor of calpains, limits pulmonary hypertension severity caused by hypoxia [15]. As the main member of calpains, calpain-1 plays an important role in cardiovascular disease and participates in the occurrence and development of vascular endothelial dysfunction [16,17]. Recent reports have also indicated that calpain-1 activation contributes to the development of PAH, and the mechanism may be related to the regulation of hypoxia inducible factor-1 (HIF-1) and NOD-like receptor thermal protein domain associated protein 3 (NLRP3) [18,19]. PD 150606, an inhibitor of calpain-1, antagonized TGF-β1-mediated EndMT in human lung epithelial A549 cells, and this effect was related to inhibition of the increased phosphoinositide-3-kinase (PI3K)/Akt signaling pathway [20]. However, how calpain-1 regulates the process of EndMT and thus regulates the development of PAH, has not been reported.

Baicalin, a flavonoid extracted from the root of *Scutellaria baicalensis* Georgi, possesses a variety of pharmacological activities, including but not limited to inducing tumor cell apoptosis [21,22]. Attenuating cardiac remodeling [23], and modulating oxidative stress and inflammation [24–26]. Some studies have shown that baicalin can improve pulmonary hypertension by stabilizing the extracellular matrix, alleviating oxidative stress, inhibiting inflammatory reactions, and then inhibiting the proliferation of smooth muscle cells [27–29]. However, it is not completely clear whether the beneficial effect of baicalin on pulmonary hypertension is attributed to the inhibition of EndMT. The aim of the present study was to evaluate the effect of baicalin on PVR with a focus on calpain-1-mediated EndMT.

2. Materials and methods

2.1. Drugs and reagents

Baicalin were obtained from Nanjing Jingzhu Biotechnology Co. Ltd. (Nanjing, China). Monocrotalin (MCT, C2401) was purchased from Sigma–Aldrich (St. Louis, MO, USA). Transforming growth factor-beta 1 (TGF-β1, HY-P70543) and calpain Inhibitor MDL-28170 (HY-18236) were purchased from MedChemExpress. Antibodies against calpain-1 (A1172), Phosphoinositide 3-kinase (PI3K, A0265), Protein kinase B (AKT, A11016), Phosphorylated protein kinase B (*p*-AKT, AP0140) were purchased from ABclonal (Wuhan, China). Antibodies against Platelet endothelial cell adhesion molecule-1 (CD31, 66065-2-Ig), Smooth muscle actin α (α-SMA, 14395-1-AP), Vimentin (10366-1-AP), VE-cadherin (66804-1-Ig) and fluorescein (FITC)-conjugated Affinipure goat anti-mouse lgG (H + L) (SA00003-1) were purchased from Proteintech (Wuhan, China). DyLight 594 AffiniPure goat Anti-rabbit IgG (H + L) (E032420-01) was purchased from EarthOx. The EdU Cell Proliferation Kit (C0071S) and Cell Counting Kit-8 (C0038) were obtained from Beyotime Biotechnology (Shanghai, China).

2.2. Animal experiments

All animal procedures were performed under the principles approved by the Animal Ethics Committee of Jinzhou Medical University (20,220,616,001). The calpain-1 gene knockout mouse is derived from the C57BL/6 N strain and is a complete knockout of the calpain-1 gene, generated by Cyagen Biosciences. C57BL/6 mice and calpain-1 gene knockout mice weighing 18–22 g were randomly assigned to the following seven groups (n = 10 per group): (a) the WT control group (Con), (b) the KO control group (KO Con), (c) the WT hypoxia group (PAH), (d) the KO hypoxia group (KO PAH), (e) the hypoxia + baicalin (40 mg/kg/day) group (PAH + Bai 40), (f) the hypoxia + baicalin (80 mg/kg/day) group (PAH + Bai 80) and (g) the hypoxia + baicalin (120 mg/kg/day) group (PAH + Bai 120). The establishment of a hypoxic pulmonary hypertension model is based on our previous report (Deng et al., 2022). Sprague Dawley (SD) rats weighing 220–250 g were randomly assigned to the following seven groups (n = 10 per group): (a) the control group (Con), (b) the MCT (60 mg/kg) group (PAH), (c) the MCT + baicalin (25 mg/kg/day) group (PAH + Bai 25), (d) the MCT + baicalin (50 mg/kg/day) group (PAH + Bai 50), (e) the MCT + baicalin (100 mg/kg/day) group (PAH + Bai 100) and (f) the MCT + MDL group (PAH + MDL). MCT was dissolved in anhydrous ethanol and normal saline at a volume ratio of 1:4 and administered intraperitoneally. Baicalin was dissolved in 0.5 % sodium carboxymethyl cellulose (CMC-Na) and administered by gavage for 4 weeks, the control group was given the same volume of sodium carboxymethyl cellulose.

2.3. HPAECs culture

Human pulmonary artery endothelial cells (HPAECs) were purchased from BLUEFBIO (Shanghai, China) and cultured in Endothelial Cell Growth Supplement (ECGS)-containing Endothelial Cell Medium (ECM) with 10 % FBS and 100 U/ml penicillin/streptomycin at 37 °C and 5 % CO_2. HPAECs at three to seven passages were divided into the following five groups: (a) the control group (Con), (b) the TGF-β1 (10 ng/ml) group, (c) the TGF-β1 + baicalin (20 μmol/L) group, (d) the TGF-β1 + baicalin (40 μmol/L) group, (e) the TGF-β1 + baicalin (80 μmol/L) group and (f) the TGF-β1 + MDL-28170 (10 μmol/L) group. Baicalin and MDL-28170 were dissolved in DMSO and added to HPAECs 30 min before TGF-β1 stimulation. After that, the HPAECs were cultured in ECM with 0.1 % FBS for 24 h.

2.4. Culture and proliferation of PASMCs

For evaluation of the effect of baicalin on PVR, pulmonary artery smooth muscle cells (PASMCs) from SD rat pulmonary arteries were used in this experiment. Briefly, SD rats were anesthetized, and the first branch of the pulmonary artery was isolated in a sterile environment. The first major branch of the pulmonary artery is also called the left superior pulmonary trunk. After the intima and adventitia were scraped, the middle membranes were cut into small pieces and washed with D-Hanks several times. The intermediate membrane is the layer of smooth muscle cells. Then, small pieces of arterial tissue (1mm × 1mm × lmm) were cultured in DMEM with 20 % FBS and 1 % penicillin/streptomycin in a humidified atmosphere of 95 % air and 5 % CO_2 at 37 °C. After 7–9 days, the cells were digested and subcultured with 0.25 % trypsin-EDTA. The cells were identified as PASMCs by immunofluorescence staining with α-SMA. The grouping and dosing were the same as those of HPAECs except that hypoxia was used instead of TGF-β1. In subsequent experiments, PASMCs were cultured in a hypoxic environment (37 °C, 3 % O_2, 5 % CO_2) to induce PASMC proliferation and migration. The activity and proliferation of PASMCs were measured by CCK-8 and EdU incorporation assays, respectively.

2.5. Weight and hemodynamic analysis

After the establishment of the pulmonary arterial hypertension model for 28 days, the weight of each group of mice or rats was determined and recorded. Then, the right external jugular vein was separated, and the right ventricular systolic pressure (RVSP), mean pulmonary artery pressure (mPAP) was measured with a pressure sensor (BL-420 S; Chengdu Taimeng SciTech Ltd.). The ratio of right ventricular weight to left ventricular + interventricular septum weight RV/(LV + s) was measured by the weighing method. Finally, the left lung tissue and left upper pulmonary artery of mice in each group were collected and stored at −80 °C for standby, and the right ventricle outside the left ventricle and heart tissue were fixed with 4 % paraformaldehyde for histological analysis.

2.6. Histological analysis

For histological analysis, the lung and heart tissues of mice or rats were separated, fixed with 4 % paraformaldehyde for 48 h, and then embedded in paraffin. The area ratio (WA%) and thickness ratio (WT%) of the vascular wall were measured according to hematoxylin eosin (H&E) staining. Cardiopulmonary fibrosis was measured according to Masson's trichrome staining. After the heart and lung tissue slices were baked, dewaxed and dehydrated routinely, Bouin solution was dripped and placed in a 37 °C water bath box for mordant dyeing for 2 h. The slices were then treated with lividin blue, mayer hematoxylin, acidic ethanol differentiation solution, carmine and phosphoric acid and finally photographed under a fluorescence microscope.

2.7. Doppler echocardiogram

Right heart function was measured using a color doppler ultrasound diagnostic system (Sigma VET; Esaote). The right and left ventricles-chamber view was used to visualize the right ventricular hypertrophy, and tricuspid annular plane systolic excursion (TAPSE) was used to evaluate right ventricular function. All tests were performed twice and analyzed by a sonographer who was blinded to the experiment.

2.8. Transwell migration assay

Transwell migration was conducted in a 24-well transwell chamber (Corning). PASMCs were digested and cultured in the upper chamber for 48 h. After PBS washes, the PASMCs were fixed with 4 % paraformaldehyde for 30 min and stained with 0.25 % crystal violet for 15 min. The upper layer cells that failed to pass through the membrane were gently removed with a cotton swab. The magnification field of view was used to count the number of migrating cells in each field of view.

2.9. Immunofluorescence

HPAECs were washed with PBS, fixed with 4 % paraformaldehyde for 30 min, infiltrated with 0.5 % Triton-X100 for 30 min and blocked with 5 % BSA for 30 min. The HPAECs were then incubated with calpain-1 antibody (1:100), HRP fluorescent secondary antibody (1:100) and DAPI. Immunofluorescence of α-SMA or calpain-1 staining of lung sections was performed using *anti*-α-SMA antibody (1:100) or *anti*-calpain-1 antibody (1:100). Then, the sectioned tissues were incubated with secondary antibodies, and DAPI

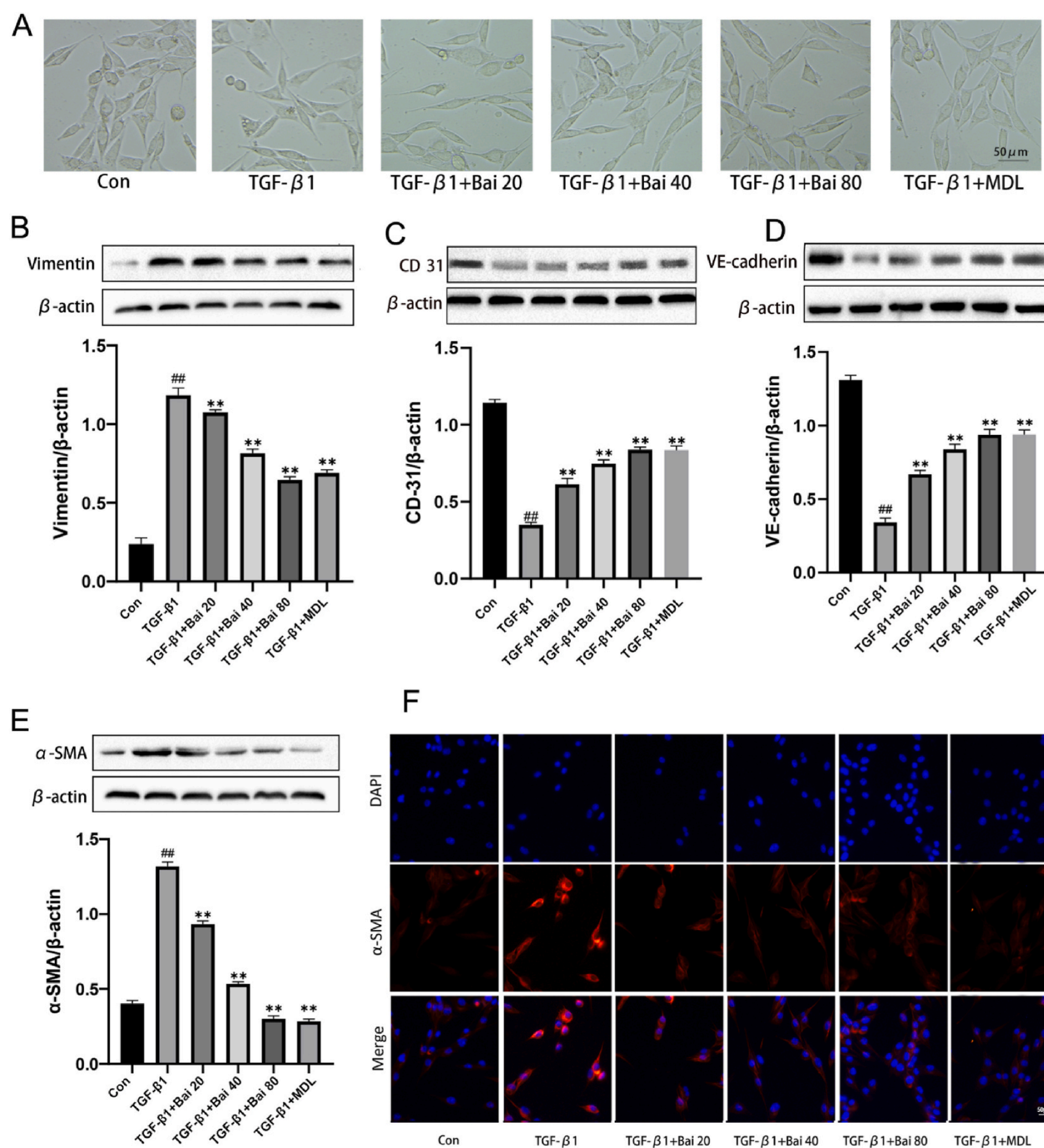

Fig. 1. Baicalin and calpain-1 inhibition prevented EndMT in HPAECs. (A) Morphological changes of HPAECs were observed under microscope. (B–E) Western blot was used to detect the expression of VE-cadherin, CD31, α-SMA and Vimentin proteins in HPAECs induced by TGF-β1. (F) Representative images of α-SMA immunofluorescence. Red fluorescence represents α-SMA, and blue is DAPI. Scale bar = 50 μm. Con: Control; Bai: Baicalin; MDL: MDL-28170. N = 3. The data are expressed as the means ± SEMs. $^{##}P < 0.01$ vs. the Con group, $^{**}P < 0.01$vs. the TGF-β1 group.

was used to stain the cell nuclei. The images were collected by fluorescence microscopy and analyzed by ImageJ software. The images were collected by fluorescence microscopy and analyzed by ImageJ software.

2.10. Western blot

The collected lung tissue and HPAECs and PASMCs were homogenized in RIPA lysis buffer. The protein concentration was measured using a BCA protein analysis kit. The samples were separated by SDS−PAGE (10 % polyacrylamide gel) and transferred to PVDF membranes. The membrane was blocked with 1 % BSA for 1 h and then incubated overnight at 4 °C with antibodies against α-SMA (1:1000), vimentin (1:1000), VE-cadherin (1:1000), CD31 (1:1000), calpain-1 (1:1000), PI3K (1:1000), AKT (1:1000), *p*-AKT (1:1000) and β-actin (1:100,000). The membrane was washed three times with TBST and then incubated with HRP (1:10,000)-bound secondary antibody at room temperature for 1.5 h. The density of protein bands was quantified by ImageJ software, and the results were normalized to β-actin.

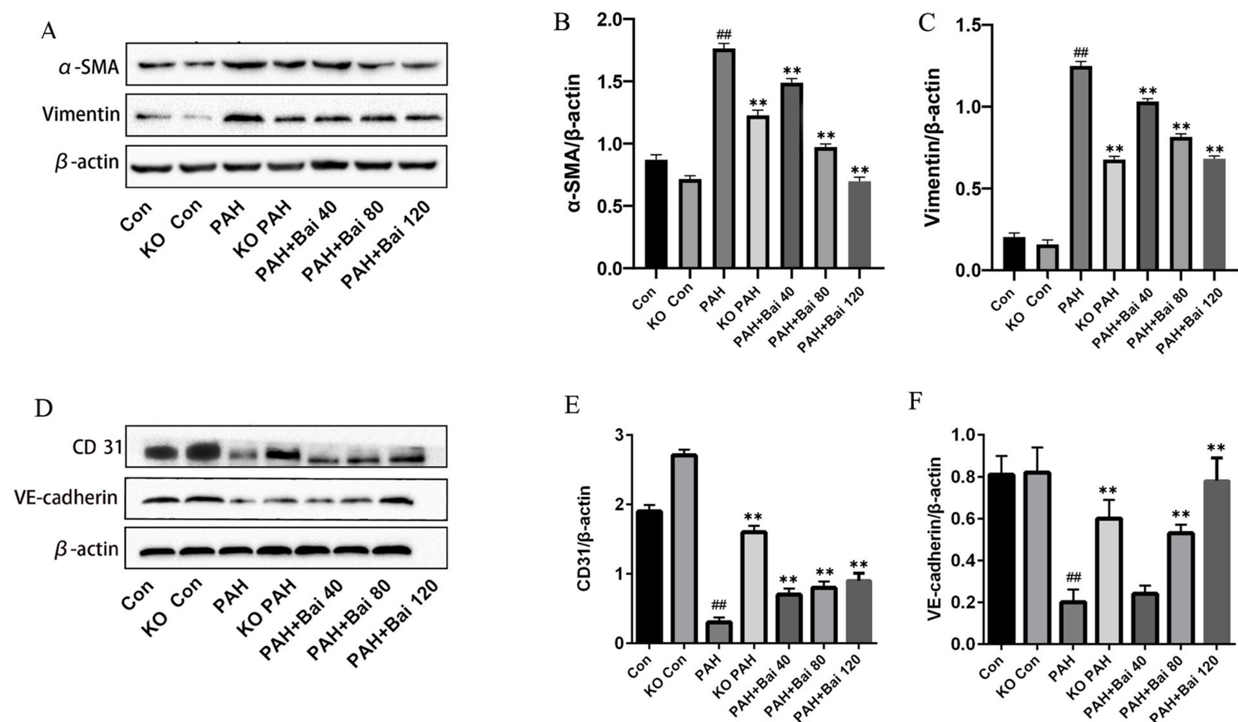

Fig. 2. Baicalin and calpain-1 inhibition prevented EndMT in mice. (A–F) Western blot was used to detect the expression of VE-cadherin, CD31, α-SMA and Vimentin proteins in the lung samples induced by hypoxia. N = 3. The data are expressed as the means ± SEM. $^{##}P < 0.01$ vs. the Con group, $^{**}P < 0.01$vs. the PAH group.

2.11. Data analysis

The results are expressed as the means ± SEMs and were analyzed by using SPSS 25.0. Univariate analysis of variance (ANOVA) was used to analyze the differences. The data were subjected to t tests. $P < 0.05$ was considered statistically significant.

3. Results

3.1. Baicalin and calpain-1 inhibition prevented EndMT in HPAECs

Previous reports have demonstrated that EndMT plays a pivotal role in PVR. To investigate the effects of baicalin and calpain-1 on EndMT, we used TGF-β1 to induce EndMT in HPAECs. As shown in Fig. 1A, after stimulation with TGF-β1 for 24 h, the cobblestone morphology of HPAECs was replaced by a spindle-shaped phenotype, indicating the development of EndMT, and the EndMT process was further confirmed by increased expression of α-SMA and vimentin and decreased expression of VE-cadherin and CD31 (Fig. 1B–E). In addition, immunofluorescence staining showed that baicalin significantly prevented the upregulation of α-SMA expression induced by TGF-β1 (Fig. 1F). MDL-28170 showed a similar effect on TGF-β1-induced EndMT as baicalin.

3.2. Baicalin and calpain-1 inhibition prevented EndMT in mice

To verify the results of the in vitro study, we established a PAH model by hypoxia in calpain-1 KO and wild-type C57BL/6 mice. As previously reported, EndMT was induced by hypoxia in mice characterized by upregulation of α-SMA and vimentin and down-regulation of VE-cadherin and CD31 (Fig. 2) in lung samples. Consistent with the in vitro results, baicalin treatment rescued these changes and weakened the EndMT process induced by hypoxia. Importantly, calpain-1 knockout reversed the EndMT process induced by hypoxia, as shown by the inhibited mesenchymal markers and increased endothelial markers.

3.3. Baicalin and calpain-1 inhibition attenuated pulmonary vascular remodeling in mice

EndMT is involved in the progression of PVR. Therefore, the current study investigated whether the inhibitory effect of baicalin and calpain-1 on EndMT contributes to their improvement in PVR and PAH. The mice in the hypoxia group showed significantly higher RVSP and RV/(LV + S) than the mice injected with saline, and the elevations in RVSP and RV/(LV + S) were repressed by baicalin and calpain-1 KO (Fig. 3A and B). Consistent with this finding, the reduction in weight gain in mice challenged with hypoxia was also

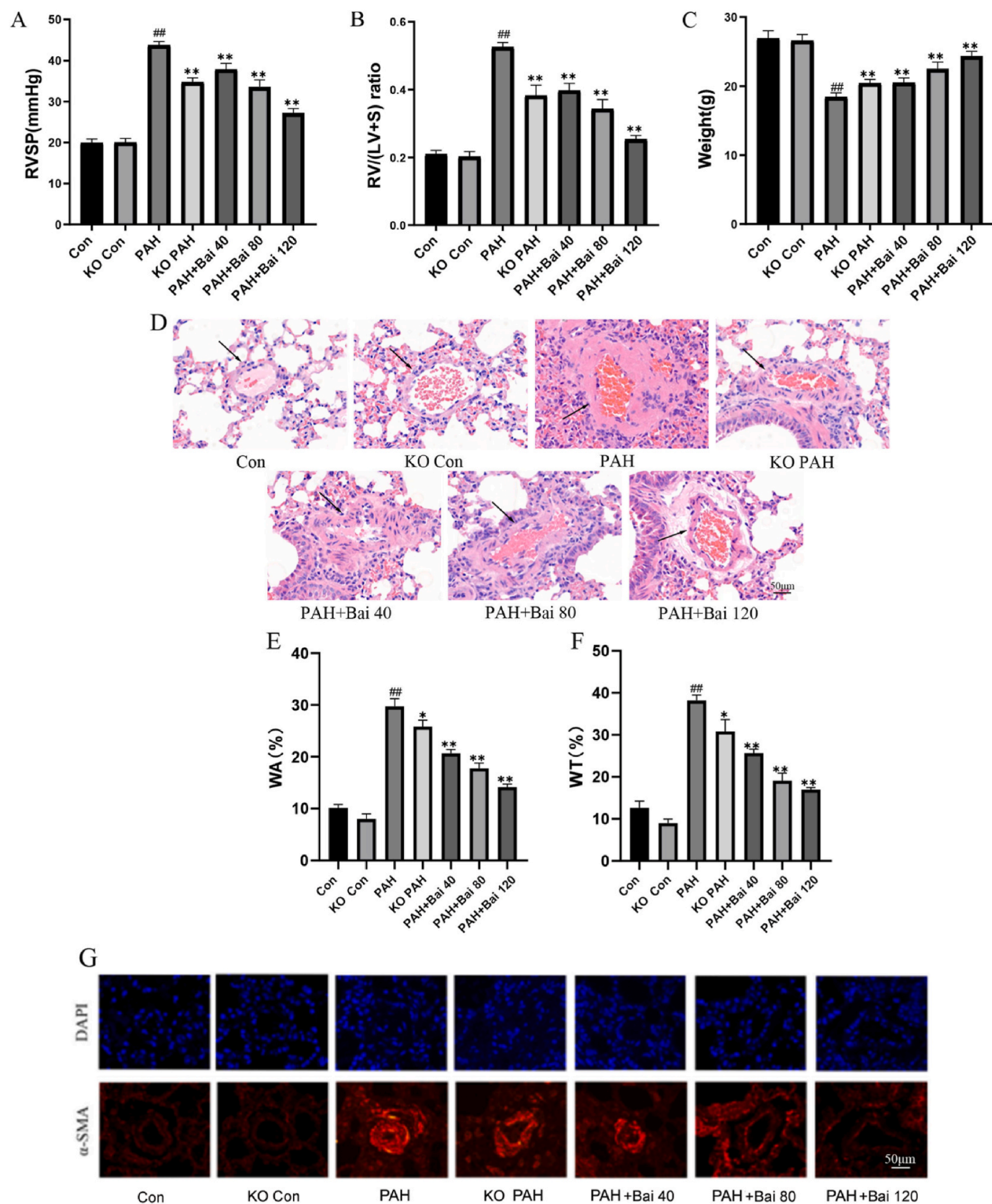

Fig. 3. Baicalin and calpain-1 inhibition attenuated PVR. (A–C) Changes of RVSP, RV/(LV + S) and body weight in mice were detected by hemodynamics and weighing methods, n = 8 for each group. (D–F) H&E staining was used to observe the vascular morphology of lung tissue, n = 4 for each group. (G) Representative images of α-SMA immunofluorescence staining, n = 4 for each group. The data are expressed as the means ± SEMs. $^{##}P < 0.01$ vs. the Con group, $^{*}P < 0.05$ vs. the PAH group, $^{**}P < 0.01$vs. the PAH group.

rescued by baicalin and calpain-1 KO (Fig. 3C). The effect of baicalin and calpain-1 KO on PVR was examined by H&E staining. As illustrated, the pulmonary artery medial wall was significantly thickened in mice with hypoxia-induced PAH. However, medial wall thickness, WT% and WA% were markedly decreased by baicalin treatment and calpain-1 KO (Fig. 3D–F). Notably, baicalin and calpain-1 KO significantly improved the muscularization induced by hypoxia, which manifested as lower expression of α-SMA in the distal pulmonary arteries (Fig. 3G).

3.4. Baicalin and calpain-1 inhibition attenuated cardiopulmonary fibrosis in mice

Masson's trichrome staining results showed that interstitial collagen deposition in the lung tissue of the hypoxic mice was

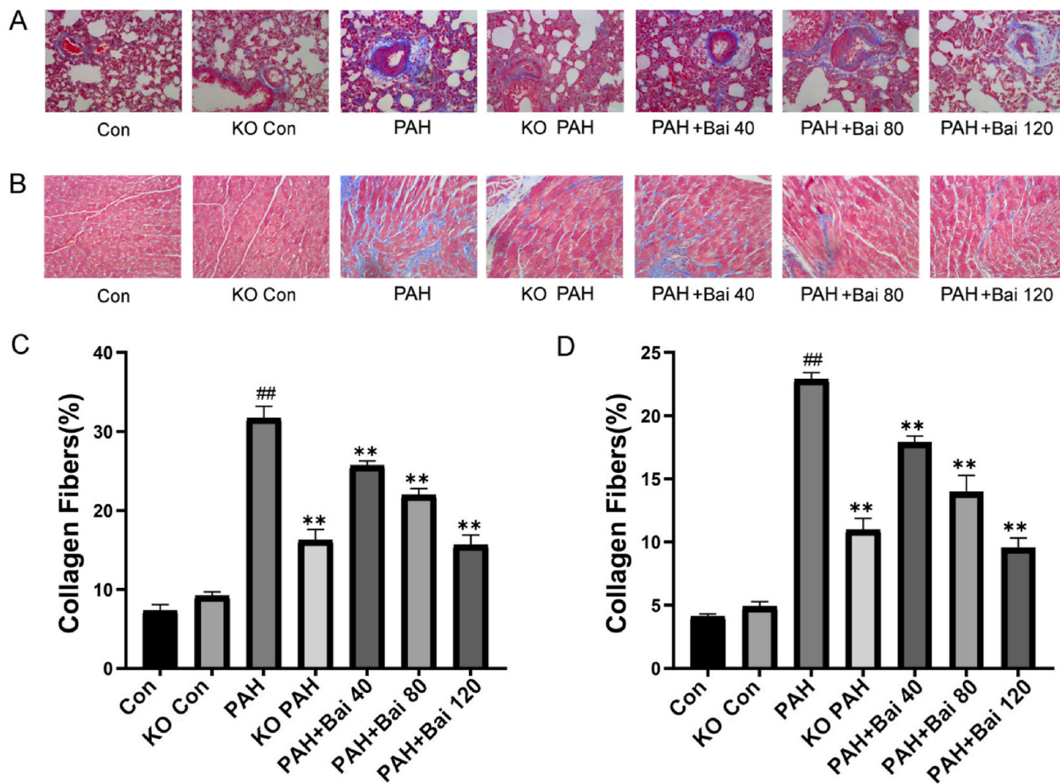

Fig. 4. Baicalin and calpain-1 inhibition attenuated cardiopulmonary fibrosis. (A–D) Cardiopulmonary fibrosis was stained by Masson trichrome.The collagen deposition and fibrosis areas are bright blue areas, n = 4 for each group. The data are expressed as the means ± SEMs. $^{##}P <$ 0.01 vs. the Con group, $^{**}P <$ 0.01vs. the PAH group.

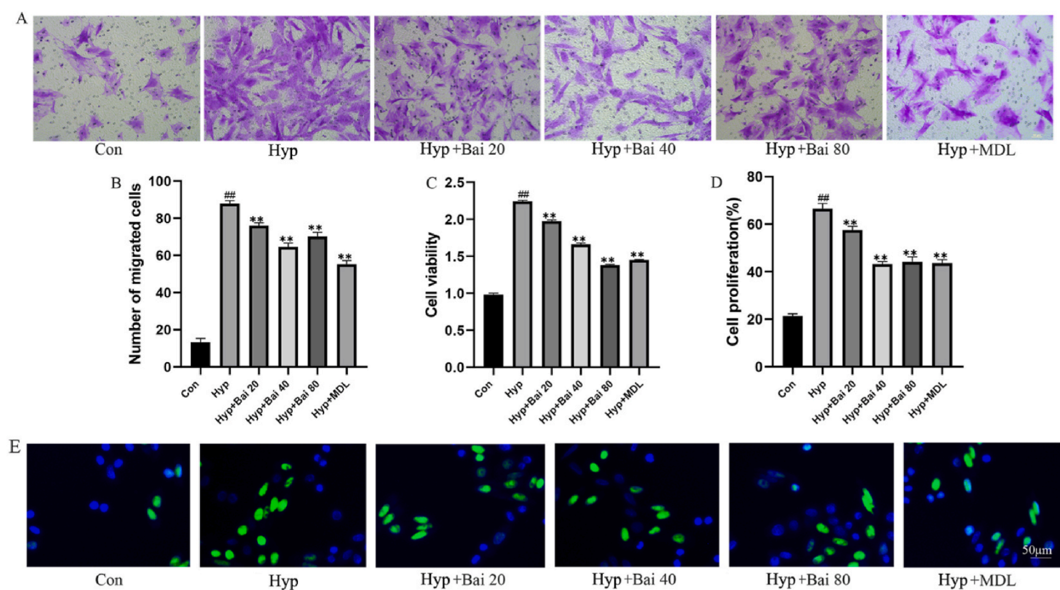

Fig. 5. Baicalin and calpain-1 inhibition attenuated proliferation and migration of PASMCs. (A–B) Transwell assay was used to detect the migration of PASMC, n = 4 for each group. (C) CCK-8 assay was used to detect cell viability. N = 3 independent experiments. (D–E) EdU incorporation was used to detect the proliferation of PASMC. n = 3 independent experiments. The data are expressed as the means ± SEMs. $^{##}P < 0.01$ vs. the Con group, $^{**}P <$ 0.01vs. the Hyp group.

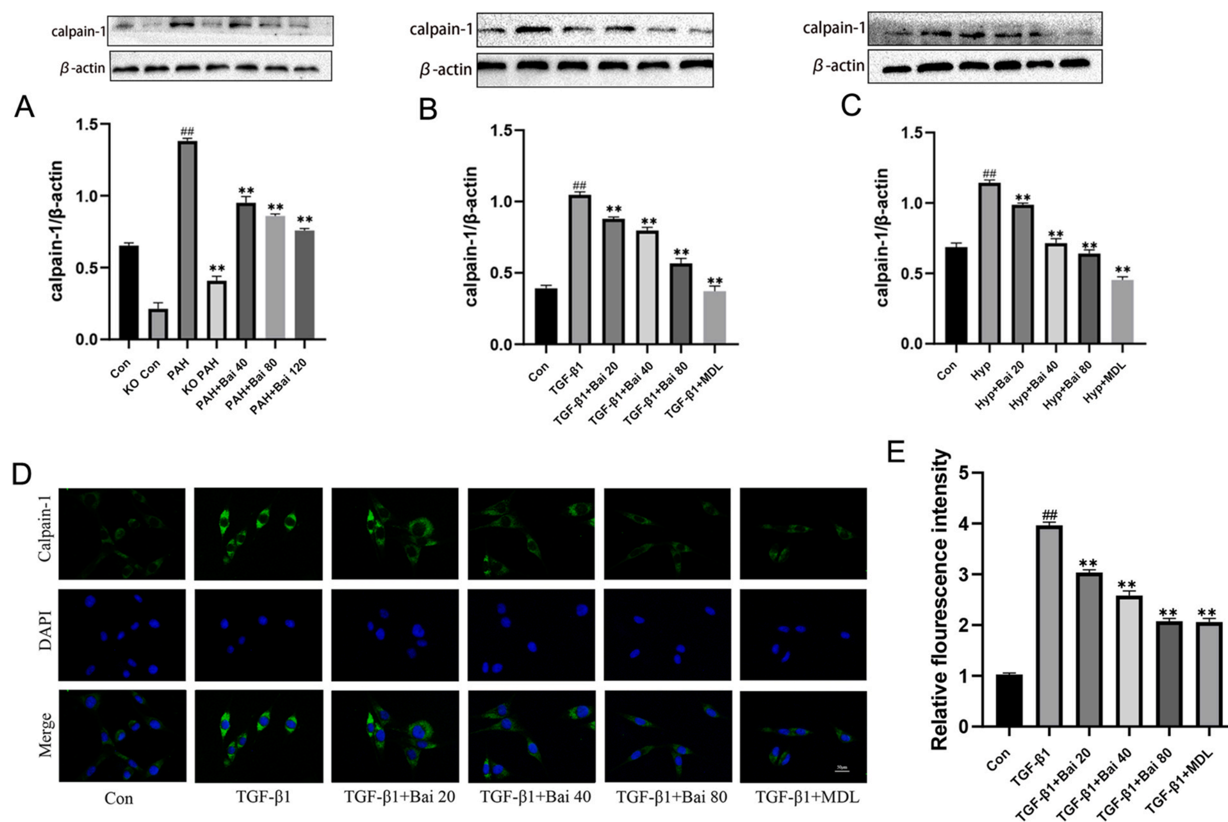

Fig. 6. Baicalin prevented EndMT process by inactivation of calpain-1. Western blot was used to detect the expression of calpain-1 protein in the lung samples(A), HPAECs(B) and PASMCs(C) induced by hypoxia, TGF-β1 and hypoxia. The expression of calpain-1 in HPAECs was detected by immunofluorescence (D-E). N = 3 for each group. The data are expressed as the means ± SEMs. $^{\#}P < 0.05$ vs. the Con group, $^{\#\#}P < 0.01$ vs. the Con group, $^{**}P < 0.01$vs. the PAH, Hyp or TGF-β1 group.

significantly higher than that of the normal saline-treated mice. Both baicalin treatment and calpain-1 KO attenuated interstitial collagen deposition in lung tissue induced by hypoxia (Fig. 4A and C). In addition, obvious interstitial fibrosis was observed in the RVs of the hypoxic mice, while baicalin treatment and calpain-1 KO attenuated interstitial fibrosis in RVs (Fig. 4B and D).

3.5. Baicalin and calpain-1 inhibition attenuated the proliferation and migration of PASMCs

Excessive proliferation and migration of PASMCs play a key role in PVR. To further explore the effect of baicalin and calpain-1 inhibition on PVR, we examined the migration and proliferation of PASMCs by Transwell assays (Fig. 5A and B), CCK-8 assays (Fig. 5C) and the EdU incorporation method (Fig. 5D and E). Not surprisingly, baicalin treatment and MDL-28170 showed similar effects on suppressing PASMC proliferation and migration induced by hypoxia.

3.6. Baicalin prevented the EndMT process by inactivating calpain-1

Since calpain-1 inhibition prevented EndMT and attenuated PVR in both in vitro and in vivo studies, the present study then investigated the role of calpain-1 in the baicalin-mediated beneficial effect on PAH. The results from the in vivo study showed that calpain-1 expression in KO mice was significantly lower than that in the Con group, indicating that calpain-1 gene knockout was successful. Calpain-1 expression in the hypoxic mice was significantly higher than that in the Con group, and this effect was abolished by baicalin treatment. In addition, upregulated calpain-1 expression was observed in both PASMCs and HPAECs, which was completely abolished by MDL-28170, and baicalin had a similar inhibitory effect on calpain-1 expression as MDL-28170 (Fig. 6A–E).

3.7. Baicalin and calpain-1 inhibition downregulated PI3K/AKT pathway

It has previously been reported that the PI3K/AKT signaling pathway is involved in the calpain-1-regulated EndMT process in human lung epithelial A549 cells. The current study then evaluated the effects of baicalin and calpain-1 inhibition on the PI3K/AKT pathway at the HPAECs and mice levels. As shown in Fig. 7 (A-C), the expression of PI3K and *p*-AKT was upregulated in the vascular tissue of hypoxic mice. Calpain-1 KO and baicalin treatment arrested the enhanced expression of PI3K and *p*-AKT. Consistent with this,

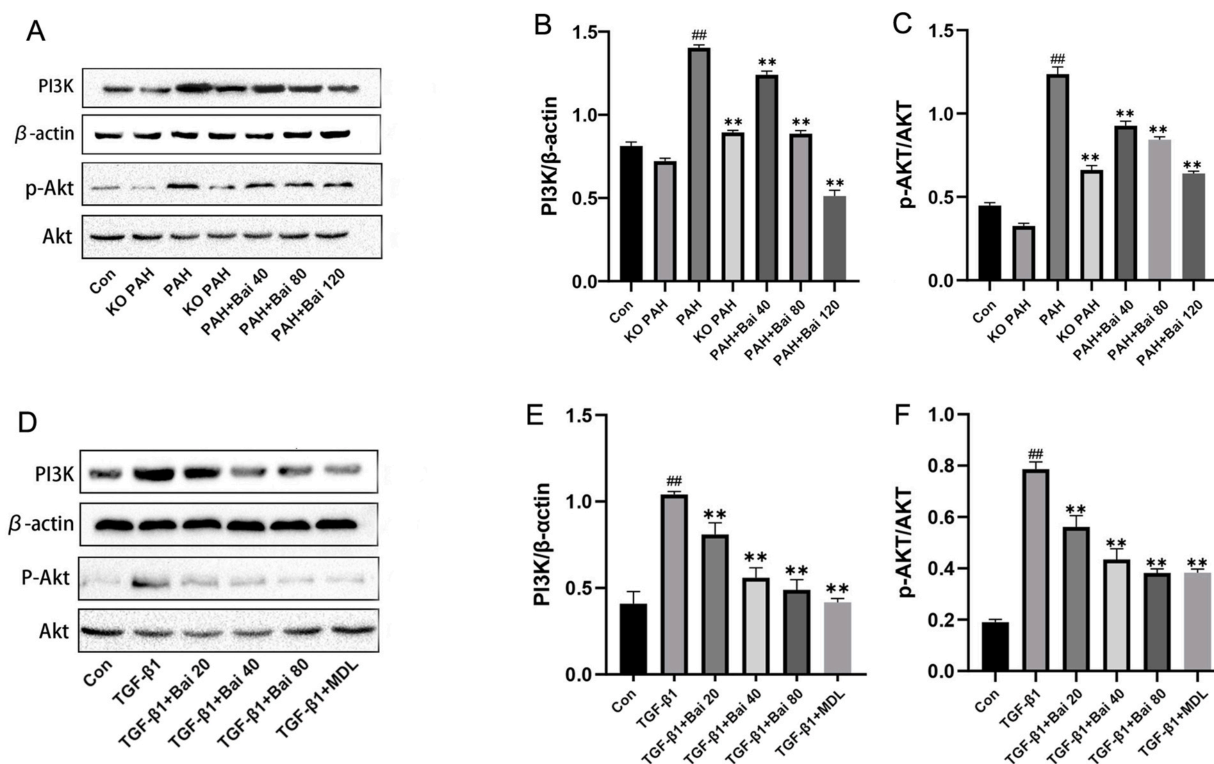

Fig. 7. Baicalin and calpain-1 inhibition downregulated PI3K/AKT pathway. Western blot was used to detect the expression of PI3K, *p*-AKT and AKT proteins in the lung samples (A-C) and HPAECs(D-F). N = 3 for each group. The data are expressed as the means ± SEMs. $^{\#\#}P < 0.01$ vs. the Con group, $^{**}P < 0.01$vs. the PAH or TGF-β1 group.

the expression of PI3K and *p*-AKT was significantly enhanced by TGF-β1 in HPAECs, while both MDL-28170 and baicalin arrested the enhanced expression of PI3K and *p*-AKT (Fig. 7D–F).

3.8. Baicalin and calpain-1 inhibition improved PAH via attenuating EndMT induced by MCT in rats

MCT is an important factor in inducing pulmonary vascular remodeling and EndMT changes. To further clarify the role of baicalin and calpain-1 inhibition in pulmonary vascular remodeling, this study induced PAH in rats by intraperitoneal injection of MCT. Four weeks after baicalin and MDL-28170 treatment, pulmonary hemodynamics, right ventricular hypertrophy and pulmonary structural changes were assessed. Hemodynamic and echocardiographic measurements demonstrated that baicalin treatment reduced PAH responses (RVSP), right ventricular hypertrophy (RV/LV + S) and right ventricular systolic function (TAPSE), while MDL-28170 showed a similar effect as baicalin (Fig. 8A,D-F). In addition, WT% and WA% were markedly decreased by baicalin and MDL-28170 treatment (Fig. 8B-C). To investigate the role of calpain-1in the improvement of PAH by baicalin, we determined the expression of calpain-1 and *p*-AKT by WB and immunofluorescence. Similar to MDL-28170, baicalin could significantly reduce the upregulation of calpain-1 and *p*-AKT expression caused by MCT, indicating the involvement of calpain-1 and *p*-AKT in the improvement of PAH by baicalin (Fig. 9F–H). Importantly, the improvement effect of baicalin on PAH was accompanied by the inhibition of the EndMT process, manifested as an increase in VE-cadherin and CD31 expression and a decrease in α-SMA and vimentin expression (Fig. 9A–E).

4. Discussion

Previous reports have demonstrated the beneficial effect of baicalin on PAH, but the mechanism is not fully understood. The current study confirmed the improvement of PVR by baicalin in PAH. The new findings of the current research are as follows: 1) The beneficial effect of baicalin on PAH is attributed to the inhibition of EndMT and PASMC proliferation. 2) Calpain-1 inhibition ameliorated the EndMT associated with PAH. 3) The improvement of PVR by baicalin is achieved at the molecular level by inhibiting the calpain-1-mediated PI3K/Akt pathway.

Recent studies have generally suggested that the EndMT process caused by endothelial injury and dysfunction is an important factor in PVR in PAH [30]. EndMT is characterized by the acquisition of mesenchymal cell markers and loss of endothelial cell markers in the endothelium. TGF-β1 stimulation, intrinsic or extrinsic factors-induced BMPR2 signaling dysfunction, and inflammatory challenge are key risk factors for EndMT in different experimental PAH models. Lung vascular endothelial cells (LVECs) isolated from patients with idiopathic pulmonary arterial hypertension (IPAH) exhibited high levels of EndMT [31]. Cumulative evidence has

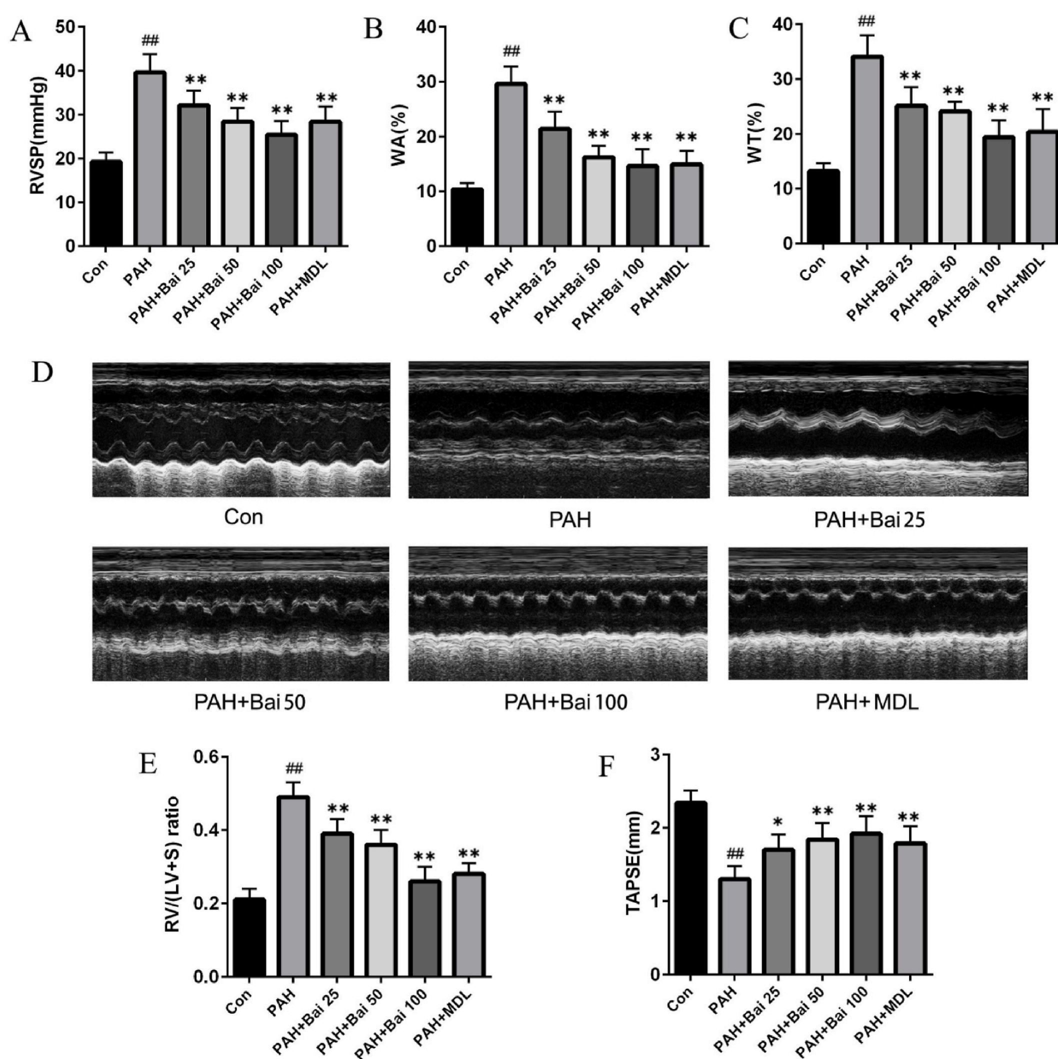

Fig. 8. Baicalin and calpain-1 inhibition attenuated PVR and right ventricular hypertrophy in rats. (A–D) RVSP, Echocardiogram, and RV/(LV + S) ratio were shown to estimate RV hypertrophy. Right ventricular function was indicated by TAPSE. (E, F) WT% and WA% were calculated, as described in materials and methods to evaluate PVR. n = 3 for each group. The data are expressed as the means ± SEMs. $^{##}P < 0.01$ vs. the Con group, $^{*}P < 0.05$,$^{**}P < 0.01$vs. the PAH group.

suggested that EndMT constitutes a potential target for experimental PAH therapy [32–34]. In the present study, HPAECs exhibited an elongated and spindle-shaped appearance instead of a cobblestone appearance after TGF-β1 challenge. Importantly, the expression of endothelial cell markers (VE-cadherin and CD31) was suppressed, while the expression of mesenchymal cell markers (α-SMA and vimentin) was increased after TGF-β1 challenge. Moreover, MCT-induced PAH in rats and hypoxia-induced PAH in mice were accompanied by EndMT. These results confirmed the previous research conclusion that EndMT could be induced by MCT or hypoxia and TGF-β1 in HPAECs. Importantly, baicalin rescued the above changes related to EndMT while improving PAH. In HPAECs, baicalin inhibited the high expression of mesenchymal markers and upregulated the low expression of endothelial markers. These results suggested that baicalin could improve PAH through regulation of the EndMT process.

Another novel finding of this study is that calpain-1 inhibition, similar to baicalin, inhibited the EndMT process of PAH accompanied by lowered RVSP and improvement in PVR and right ventricle fibrosis induced by MCT or hypoxia. In addition, MDL-28170, an inhibitor of calpain-1, ameliorated EndMT induced by TGF-β1 in HPAECs, which was related to the inhibition of hypoxia-induced proliferation and migration of PASMCs. The results from previous report showed that MDL-28170 reduced the incidence of hemodynamic instability, preserved the abundance and organization of the focal adhesion protein talin, and subsequently improved right ventricle function in a pig acute pulmonary hypertension model [35]. Baicalin has protective effect on pulmonary hypertension [36, 37]. Consistent with current research, we and other laboratories have confirmed the involvement of calpain-1 in pulmonary hypertension [38,39], and the mechanism may be related to the regulation of TGF-β1, HIF-1 and NLRP3. EndMT is involved not only in pulmonary hypertension but also in other cardiovascular diseases. In an experimental idiopathic pulmonary fibrosis model, inhibition

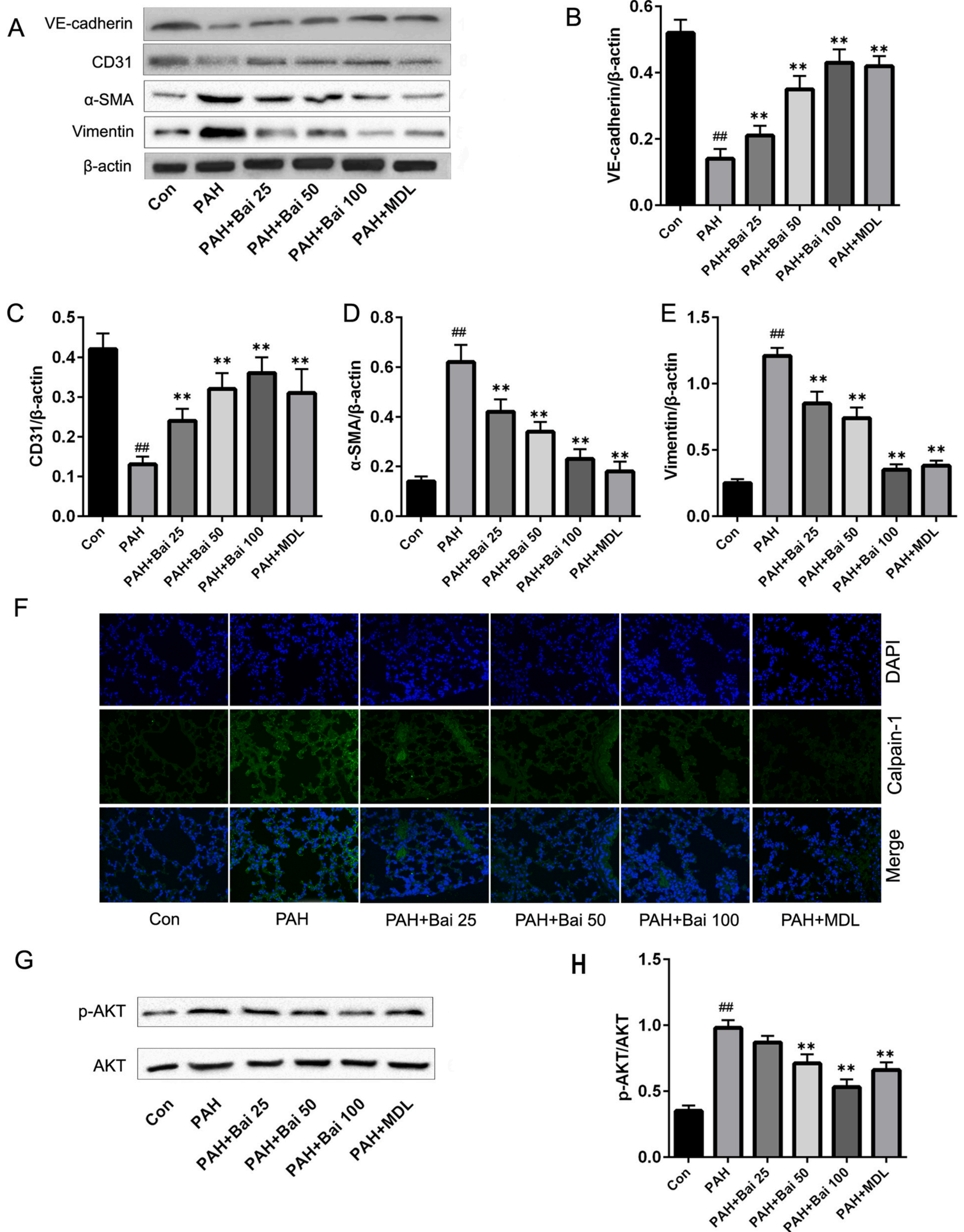

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Fig. 9. Baicalin and calpain-1 inhibition prevented EndMT in rats. (A–E) Western blot was used to detect the expression of VE-cadherin, CD31, α-SMA and Vimentin proteins in the lung samples of rats induced by MCT. (F) The expression of calpain-1 was detected by immunofluorescence. (G) Western blot was used to detect the expression of AKT and *p*-AKT. n = 3. The data are expressed as the means ± SEMs. $^{\#\#}P < 0.01$ vs. the Con group, $^{**}P < 0.01$vs. the PAH group.

of calpain-1 with an exogenous inhibitor regulated EndMT in the epithelial A549 cell line [20]. Capn4 KO specific to ECs attenuated cardiac fibrosis and dysfunction induced by ISO, and this effect was related to lowered calpain-1 activity and EndMT. Consistently, the present study confirmed the regulatory effect of calpain-1 on EndMT in a PAH model.

The PI3K/Akt pathway participates in the EndMT process in different disease states and shows different or even opposite trends. In the present study, the expression of PI3K and *p*-Akt was increased in hypoxia-exposed mice and TGF-β1-stimulated HPAECs, which was consistent with some previous reports of PAH [40–42]. In the experimental PAH model induced by hypoxia and MCT, the PI3K/Akt pathway is activated and has also become the target of some *anti*-PAH agents. Additionally, in a model of idiopathic pulmonary fibrosis and myocardial hypertrophy, the regulation of the EndMT process by calpain-1 is related to the PI3K/AKT pathway [20]. Although the PI3K/Akt pathway was activated in hypoxic mice, the calpain-1 KO mice showed lower expression of PI3K and *p*-Akt, suggesting the regulatory effect of calpain-1 on the PI3K/Akt pathway in hypoxia-induced PAH. Importantly, the improvement of PVR by baicalin was related to the inhibition of calpain-1 and the PI3K/Akt pathway. In addition, the improvement of baicalin on PVR is dose-dependent on the inhibition of calpain-1 and PI3K/Akt pathway. This experiment still has some limitations, different doses of baicalin have different effects, so it is still necessary to continue to explore the optimal therapeutic endpoint of baicalin. Further research on the Calpain-1 pathway is still needed.

In conclusion, our data in the present study demonstrated the therapeutic potential of baicalin in PAH. Baicalin treatment improved hemodynamic and right heart function and reduced cardiopulmonary fibrosis. The beneficial effect of baicalin on PAH may be attributed to the inhibition of EndMT and PASMC proliferation, and the mechanism was related to inactivation of calpain-1 and the PI3K/Akt pathway.

Data availability statement

Data will be made available on request.

Ethics declarations

This study was reviewed and approved by [Animal Ethics Committee of Jinzhou Medical University], with the approval number: [20,220,616,001].

CRediT authorship contribution statement

He-xi Jiang: Writing – original draft, Methodology, Data curation. **Xiao-di Wang:** Validation, Methodology, Investigation. **Hong-xin Wang:** Writing – review & editing, Resources. **Tong Liu:** Writing – review & editing, Resources.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e23076.

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