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Research Article

Ginsenoside Rg1 alleviates vascular remodeling in hypoxia-induced pulmonary hypertension mice through the calpain-1/STAT3 signaling pathway

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ABSTRACT

Background: Hypoxic pulmonary hypertension (HPH) is the main pathological change in vascular remodeling, a complex cardiopulmonary disease caused by hypoxia. Some research results have shown that ginsenoside Rg1 (Rg1) can improve vascular remodeling, but the effect and mechanism of Rg1 on hypoxia-induced pulmonary hypertension are not clear. The purpose of this study was to discuss the potential mechanism of action of Rg1 on HPH.

Methods: C57BL/6 mice, calpain-1 knockout mice and Pulmonary artery smooth muscle cells (PASMCs) were exposed to a low oxygen environment with or without different treatments. The effect of Rg1 and calpain-1 silencing on inflammation, fibrosis, proliferation and the protein expression levels of calpain-1, STAT3 and p-STAT3 were determined at the animal and cellular levels.

Results: At the mouse and cellular levels, hypoxia promotes inflammation, fibrosis, and cell proliferation, and the expression of calpain-1 and p-STAT3 is also increased. Ginsenoside Rg1 administration and calpain-1 knockdown, MDL-28170, and HY-13818 treatment showed protective effects on hypoxia-induced inflammation, fibrosis, and cell proliferation, which may be associated with the downregulation of calpain-1 and p-STAT3 expression in mice and cells. In addition, overexpression of calpain 1 increased p-STAT3 expression, accelerating the onset of inflammation, fibrosis and cell proliferation in hypoxic PASMCs.

Conclusion: Ginsenoside Rg1 may ameliorate hypoxia-induced pulmonary vascular remodeling by suppressing the calpain-1/STAT3 signaling pathway.

1. Introduction

HPH is a progressive cardiopulmonary illness caused by continuous hypoxic exposure [1]. As a result of HPH, pulmonary vascular remodeling occurs, including fibrosis in the arteries, inflammation, hyperproliferation and abnormal apoptosis [2]. In HPH, the walls of the pulmonary vessels thicken, which can narrow lumen infiltration, increase resistance to pulmonary blood flow, and ultimately lead to right heart failure or even death [3]. Vascular structural changes are the main underlying cause of HPH [4]. HPH pathogenesis is complicated, has not been fully elucidated, and there are no effective clinical treatment methods. Thus, it is important to find new therapeutic drugs and reveal the underlying molecular mechanisms.

The STAT3 (signal transduction and transcriptional activator 3) protein family members are transducers of many important cytokines and growth factors [5]. Currently, it is widely believed that STAT3 is an IL-6 downstream target, through the tyrosine phosphorylation of 705, the cytoplasm to the nucleus shuttle, and the STAT3-specific DNA base sequence of cognition; then, it participates in proliferation, apoptosis, fibrosis and angiogenesis of the target genes of transcription activation and induction of STAT3 activation [6,7]. STAT3 is an important signaling protein that promotes fibrosis. It is associated with fibroblast

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activation, proliferation and extracellular matrix (ECM) deposition. Specifically, in fibrogenesis, TGF-β promotes the phosphorylation of STAT3 and its transfer to the nucleus for further gene transcription [8]. Other studies have found that in PASMCs, hypoxia can activate the STAT3 pathway and promote cell proliferation [9]. In addition, studies have shown that STAT3 is a substrate for calpain-1 both in vitro and in vivo [10].

Calpain-1 belongs to the nonlysosomal cysteine protease family, which is activated mainly by increasing intracellular Ca^{2+} concentration and is strongly associated with many chronic vascular diseases [11,12]. Calpain-1 has been found to be remarkably upregulated in the lung tissue of mice under oxygen deprivation conditions and is involved in the occurrence of HPH [13]. Furthermore, some studies have further found that calpain mediates type I collagen synthesis by promoting TGF-β1 synthesis and activation in pulmonary fibrosis induced by bleomycin [14]. However, the action of the calpain-1/STAT3 signaling pathway in HPH remains unclear.

Ginsenoside Rg1 (Rg1) is considered a major active ingredient purified from ginseng. It belongs to the ginsenosides and is a monomonoside component with high content in panax notoginseng total saponins. Its pharmacological activity is extensive and has an impact on the cardiovascular system, nervous system and immune system [15]. Ginsenoside Rg1 has been reported to improve myocardial remodeling and reduce cardiac injury in thromboembolic pulmonary hypertension [16]. In addition, our previous studies have shown that Rg1 improves endothelial-to-mesenchymal transition (EndMT) and inflammatory responses in hypoxic pulmonary hypertension (HPH) rats and vascular endothelial dysfunction due to chronic intermittent hypoxia [17,18]. However, the potential mechanism of Rg1 in hypoxia-induced pulmonary hypertension is not yet clear. Therefore, we silenced calpain-1 in vivo and in vitro to further explore the role of Rg1 in HPH and its possible mechanism.

2. Materials and methods

2.1. Animal experiments

All animal experiments in this research were conducted under the Animal Experiment Ethical Commission of Jinzhou Medical University (2021018). Mice (from Liaoning Changsheng Biotechnology Company Limited) were maintained in a regulated environment for one week (free access to food and water, 25 ± 2 °C). Fifty male wild-type C57BL/6 mice were randomized into five groups: normoxia group (WT Nor), hypoxia group (WT Hyp), Hyp + ginsenoside Rg1 (Nanjing Jingzhu Bio-Technology Co.) low dose group (RL, 5 mg/kg/d), Rg1 medium dose group (RM, 10 mg/kg/d) and Rg1 high dose group (RH, 20 mg/kg/d). Twenty male calpain-1 knockout mice (Capn1 EK684−/−; Cyagen Biosciences co., Ltd.) were randomized into two groups: normoxic knockout group (KO Nor) and hypoxia knockout group (KO Hyp). The normoxic group were subjected to normal conditions with 21% oxygen, and the hypoxic group were subjected to a normal environment containing 10% oxygen for four weeks [13]. During the hypoxia period, ginsenoside Rg1 groups were intragastrically administered ginsenoside Rg1 (dissolved in 0.5% carboxymethyl cellulose) at 5, 10 or 20 mg/kg daily for four weeks. Four weeks later, mice were injected intraperitoneally with 20% urethane anesthesia. The removed heart and lung tissues and serum were kept in a −80 °C refrigerator for subsequent experimentation.

2.2. Cell culture

Healthy SPF grade Sprague Dawley (SD) rats weighing approximately 180 g were injected intraperitoneally with 20% sterile urethane. Disinfect by immersion in 75% alcohol. Rats were fixed and transferred to a sterile operating room. From the right ventricle, arterioles separate from the lung. Fibrous tissue and adipose tissue were removed from the adventitia of the pulmonary artery with curved microforceps, and the vasculature was incised longitudinally to expose the intimal surface of the vessels and remove vascular endothelial cells. The remaining tissue was cut into 1-mm square fragments and cultured in cell culture flasks, which were incubated in DMEM (Gibco) with 20% FBS (Sigma) and 1% penicillin/streptomycin and then placed in a cell incubator with 5% CO_2 at 37 °C. In three to five days, cells can crawl out around the tissue blocks. The medium was changed every 3 days. After 8–10 days, digestion and subculturing were performed with 0.25% trypsin-EDTA. Immunofluorescence staining with α-smooth muscle actin (α-SMA) was used to identify PASMCs. The next cell experiment grouped PASMCs into five groups: normoxia (Nor), hypoxia (Hyp), hypoxia + MDL-28170 (Hyp + MDL, calpain-1 inhibitor, MCE, New Jersey, USA, 10 μmol/L), hypoxia + HY-13818 (Hyp + HY, STAT3 inhibitor, MCE, New Jersey, USA, 10 μmol/L) and hypoxia + Rg1 (Hyp + Rg1, 20 μM). Cells in the hypoxic group were cultured in incubators with low oxygen concentrations set in advance (37 °C, 3% O_2, 5% CO_2). To investigate the underlying mechanisms of calpain-1 regulation of HPH, we obtained the capn1-lentiviral vector (pLVCAPN1, GV367) from GeneChem (Shanghai, China). It was incorporated into PASMCs for 12 h, and then the transfected cells were placed under hypoxic conditions for 24 h. Western blot analysis confirmed the effectiveness of overexpression.

2.3. Echocardiography

Right heart function was measured using a color Doppler ultrasound diagnostic system (Sigma VET: Esaote). The mice breathed spontaneously after anesthesia. The left side of the chest was scraped flat, and an acoustically coupled gel was used to increase probe access. Keeping the room temperature at approximately 25 °C, hypothermia was avoided in mice. The use of high resolution ultrasound imaging system to evaluate cardiac function.

2.4. Hemodynamic measurements

Mice were anesthetized with 20% urethane, and hemodynamic measurements were performed. The right jugular vein was carefully separated, and blood return was blocked with a thin line. Right ventricular systolic pressure (RVSP) and mean pulmonary artery pressure (mPAP) were measured by slowly connecting the BL-420S Biofunctional Experiment System (Chengdu Tai Meng Technology Co., Ltd.) through the right external jugular vein incision. Next, the left lung was immobilized in 4% paraformaldehyde for subsequent experiments, and the right lung was rapidly frozen. The hearts of mice were removed after thorax opening, and the blood was washed with PBS. The division of the heart into two parts, the right ventricle and the left ventricle + ventricular septum (RV/(LV + S)), which were, respectively called dry weight. The index of right ventricular hypertrophy RV/(LV + S) was used to calculate and evaluate the degree of right ventricular hypertrophy [19].

2.5. Histology analysis

Lung tissue was fixed with 4% paraformaldehyde for 48 h. After dehydration with an ethanol gradient, and after embedding the lung tissue in paraffin wax, slices of 5 μm thick were cut off. The sections were used for Hematoxylin-eosinstaining (HE) staining and Masson's trichrome staining, and the changes in pulmonary vascular morphology and collagen fiber deposition area were observed under an optical microscope (Leica DMI 3000B). ImageJ was used to compute pulmonary artery wall area/total vessel area (WA/TA) and vessel wall diameter/total vessel wall diameter (WT/TT). Lung tissue collagen fiber deposition index = (area of collagen deposition/total area) × 100%.

2.6. Immunohistochemistry and immunofluorescence staining

The phosphorylation levels of Proliferating Cell Nuclear Antigen (PCNA), Ki-67 and STAT3 (p-STAT3) were detected by immunohistochemical staining. Simply put, the sections were deparaffinized and antigen-repaired and incubated overnight at 4 °C with antibodies against PCNA, Ki-67 (from AB clonal, Wuhan, China) and p-STAT3 at a 1:100 dilution. After washing each lung section with PBS, the secondary antibody conjugated with horseradish peroxidase were incubated for 20 min at 37 °C, and then stained with DAB solution for 2–5 min. Then, the sections were washed in distilled water and reverse stained with hematoxylin at room temperature for 3 min. The sections were observed and photographed under a Leica microscope. The levels of calpain-1 and p-STAT3 in lung tissues and PASMCs were measured by immunofluorescence staining. Next, the sections and cells were fixed with 4% paraformaldehyde for 30 min, soaked with 0.5% Triton for 20 min, and then sealed with 5% BSA for another 30 min. The sections and cells were then incubated overnight with an anti-P-STAT3 (1:1000) and anti-calpain-1 (1:1000) primary antibody at 4 °C. The cells were then incubated with anti-rabbit FITC (1:100) for 1 h at 37 °C under protection from light. DAPI was used to label the nuclei for 5 min. Images were collected by fluorescence microscopy.

2.7. Enzyme-linked immunosorbent assay

After collecting blood from the eyeballs of the mice, the samples were centrifuged at 3600 r/min for 5 min, and the upper serum was taken and stored for detection by the kit. The levels of IL-6 and TNF-α in mouse serum and supernatants of PASMCs were detected by ELISA kits (from AB clonal, Wuhan, China). Hydroxyproline (HYP) ELISA kit was used to detect the levels of HYP in mouse serum and lung tissue homogenates. The assay was performed according to the manufacturer's instructions.

2.8. Western blot assay

Lung tissue and PASMCs were added with RIPA strong lysate (containing 1% PMSF), which was cracked on ice to extract total protein. After quantification of BCA protein, the sample was prepared. The sample was placed in an 8%~10% SDS−PAGE gel, and then a half dry membrane transfer instrument was used to transfer the protein on the gel onto a PVDF membrane. PVDF membrane was added to 1% BSA in a room temperature shaker and sealed for 1.5 h, then calpain-1, STAT3, p-STAT3(from Proteintech, Wuhan, China), TGF-β1, VCAM-1 (from ABclonal, Wuhan, China), ICAM-1, IL-6 and PCNA (from Proteintech, Wuhan, China) were added as primary antibody, and incubated in a refrigerator at 4 °C overnight. On the next day, TBST was washed 3 times, and the membranes were incubated with HRP-conjugated secondary antibodies for 1 h. The blots were visualized using chemiluminescence systems, and a gel imager was used for detection and analysis. Then, each band was analyzed using ImageJ software.

2.9. Cell viability assay

The viability of PASMCs was evaluated using the cell counting kit-8 (CCK-8 kit). Simply put, PASMCs were inoculated in a 96-well plate at a density of 2×10^4 cells/well at a given time, and under different conditions, 10 μl reagent was added for 1 h, and the absorbance at 450 nm was determined by an enzyme-labeled apparatus (Bio-Rad, USA).

2.10. Cell proliferation assay

PASMCs were incubated in 96-well plates in a hypoxic or normoxic culture chamber for 24 h. The 5-ethynyl-2′-deoxyuridine (EdU) kit (Beyotime) was used according to the instructions to detect cells proliferation.

2.11. Statistical analysis

SPSS software was used to process the result data, and all data are presented as the mean ± SD. GraphPad Prism (version 8.0.2) was used to perform the analysis of the experimental data. Comparisons among groups were performed using one-way analysis of variance (ANOVA) followed by Tukey's test. Differences were considered statistically significant at $P < 0.05$.

3. Results

3.1. Effects of ginsenoside Rg1 and Capn1 gene knockout improve hypoxia-induced PAH

To investigate the effect of Rg1 administration and calpain-1 knockout on HPH, hemodynamic studies showed that mPAP (Fig. 1A) and RVSP (Fig. 1B) were noticeably increased in mice with 4 weeks of hypoxia, and Rg1 treatment effectively reduced the hypoxia-induced elevation of mPAP and RVSP. Consistent with this, echocardiographic analysis showed a significantly lower decrease in PAT/PET in the WT Hyp group than in the WT Nor group. However, PAT/PET was elevated to varying degrees in both the Rg1-treated and KO Hyp groups (Fig. 1C–F). As shown in Fig. 1D and G, (RV/LV + VS) and representative right ventricular morphology M-mode echocardiography showed significant right ventricular thickening in the WT Hyp group compared with the WT Nor group, while Rg1 administration treatment and KO Hyp group showed significant improvements. B-mode echocardiography showed right ventricular hypertrophy and increased right ventricular area in the WT Hyp group compared with the WT Nor group (Fig. 1E–H), and RVFAC was improved to varying degrees in both the Rg1 and KO Hyp groups. The results suggested that Rg1 administration and calpain-1 knockdown could improve right ventricular pressure elevation and right ventricular hypertrophy in HPH mice.

3.2. Effects of ginsenoside Rg1 and Capn1 knockout on pulmonary vascular remodeling in HPH mice

As shown in Fig. 1I, the pulmonary artery wall was significantly thickened and the lumen was narrowed in the WT Hyp group compared with the WT Nor group. Western blotting was used to detect PCNA expression in the lung tissue of mice. Ki-67 and PCNA were analyzed in paraffin-embedded mouse lung tissue. Compared with the WT Nor group, WA (%) (Fig. 1J), WT (%) (Fig. 1K), PCNA and Ki-67 in lung tissue of the WT Hyp group were obviously higher compared with the WT Nor group. However, the Rg1 treatment group and KO Hyp group obviously alleviated the aforementioned changes in HPH mice (Fig. 2A–D, Fig. 3A–B). The above results indicated that Rg1 treatment and Capn1 knockout could reduce pulmonary vascular remodeling in the lung tissue of HPH mice.

3.3. Effects of ginsenoside Rg1 and Capn1 knockout on pulmonary fibrosis in HPH mice

Masson's trichrome staining was used to observe the deposition of collagen fibers in lung tissue. As shown in Fig. 2 E and F, the collagen fiber deposition area of mice in the WT Hyp group was obviously increased compared to that of mice in the WT Nor group. The collagen fiber deposition area of the Rg1 treatment group and KO Hyp group was significantly reduced. In addition, α-SMA was analyzed by paraffin-embedded mouse lung tissue, and the HYP of serum and lung homogenate was detected by ELISA. Compared with the WT Nor group, the expression levels of α-SMA, TGF-β1, Collagen I and HYP were significantly increased in the lung tissue of the WT Hyp group. Rg1 administration and calpain-1 knockout dramatically relieved these changes in HPH mice (Fig. 2 G-J, Fig. 3C–E). Meanwhile, as shown in Fig. 2K, compared with the WT Nor group, the lung dry−wet weight ratio (W/D

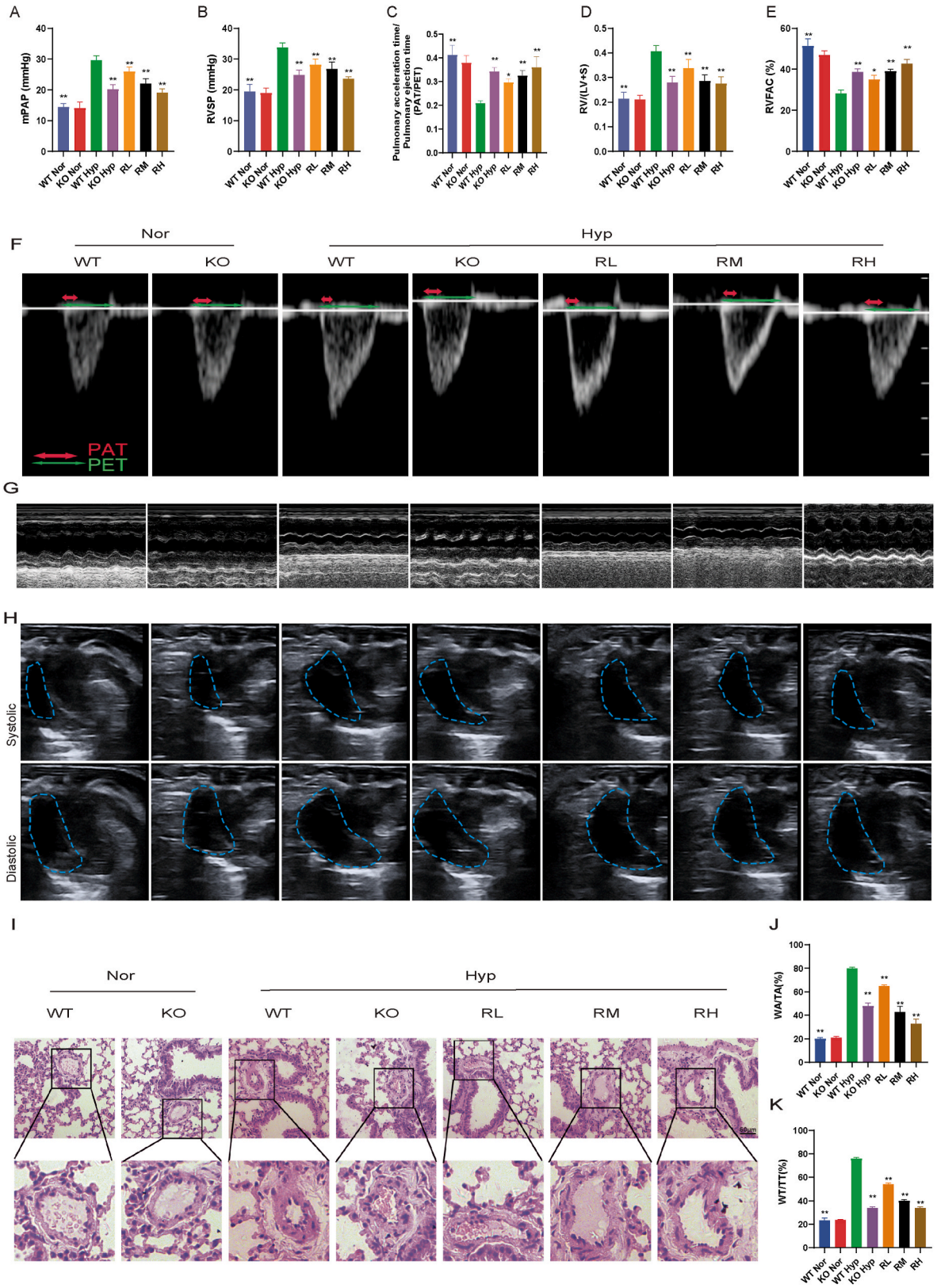

Fig. 1. Ginsenoside Rg1 and Calpain-1 Knockout improve hypoxia-induced pulmonary hypertension. (A) mPAP and (B) RVSP in various groups of mice (n = 8). (C) The ratio of the acceleration time and ejection time in pulmonary tissue(n = 3). (D) The RV/(LV + S) (n = 8). (E) Statistical data of RVFAC in each group [RVFAC= (right ventricular end diastolic area - right ventricular end systolic area)/right ventricular end diastolic area × 100%], (n = 3). (F) Representative pulse wave echocardiogram of pulmonary blood flow (n = 3). (G)Representative M-mode echocardiography of right ventricular morphology (n = 3). (H) Representative B-mode apical four-chamber right ventricular end-diastolic and end-systolic area measurements in each group (n = 3). (I) Observation of vascular morphology in lung tissue by HE staining (n = 3). (J) (K) The values of WA% and WT% were analyzed (n = 8). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with WT Hyp group).

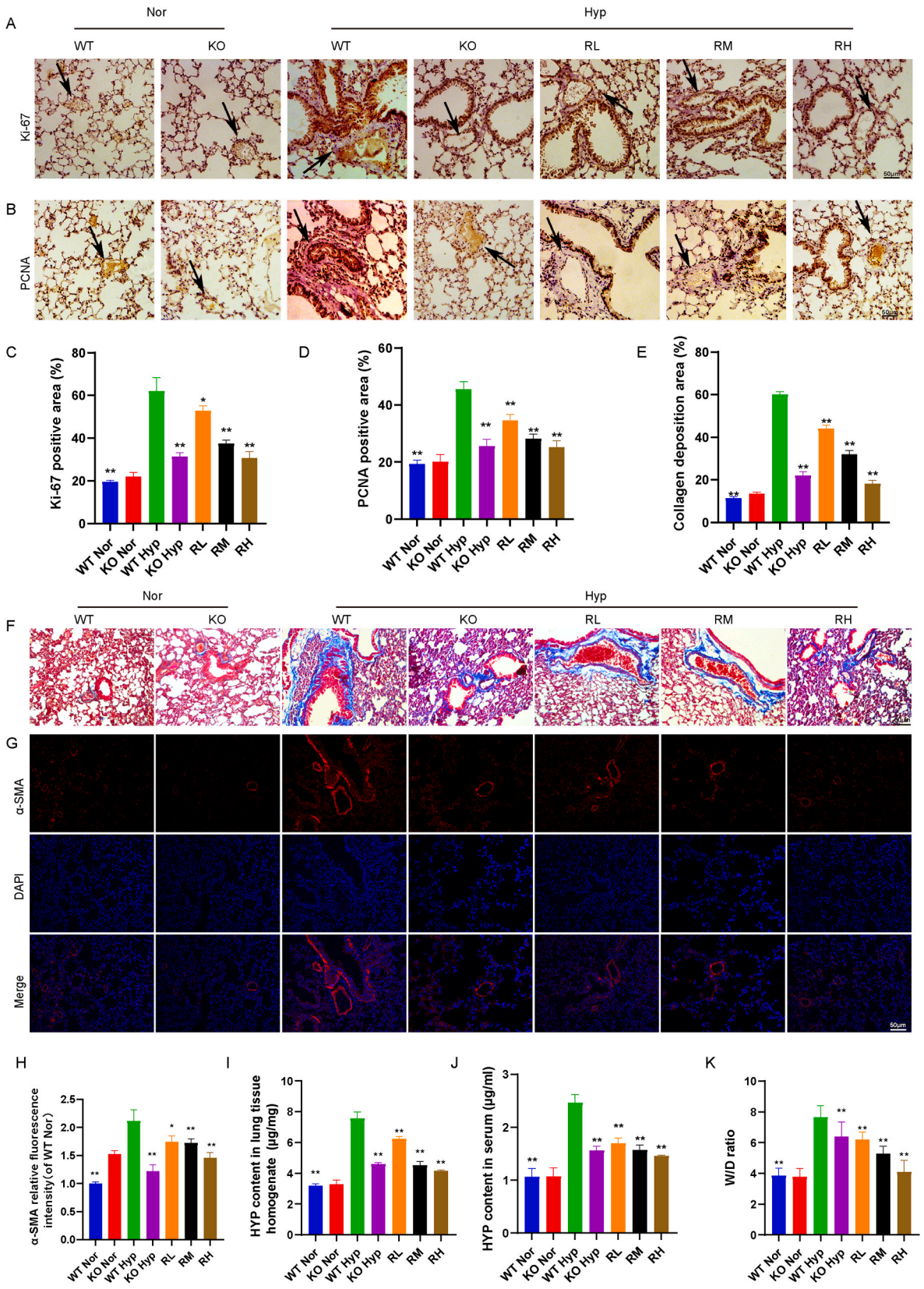

Fig. 2. Ginsenoside Rg1 and Calpain-1 Knockout improve hypoxia-induced pulmonary vascular remodeling and fibrosis. (A) (B) Immunohistochemical staining detection of Ki - 67 and PCNA expression in lung tissue (n = 3). (C) (D)The proportion of Ki-67 and PCNA positive cells ascertained by immunohistochemical staining. (E) Statistical analysis of the collagen fiber deposition area in the lung tissue of mice. (F) Masson's trichrome staining was used to test for pulmonary fibrosis (n = 3). (G) Immunofluorescence staining for α-SMA expression in lung tissue (n = 3). (H) Statistical analysis of the relative intensity of α-SMA immunofluorescence (of WT Nor). (I) (J)HYP levels in serum and lung homogenate were determined by ELISA (n = 8). (K) The W/D ratio of mouse lung tissue (n = 8). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with WT Hyp group).

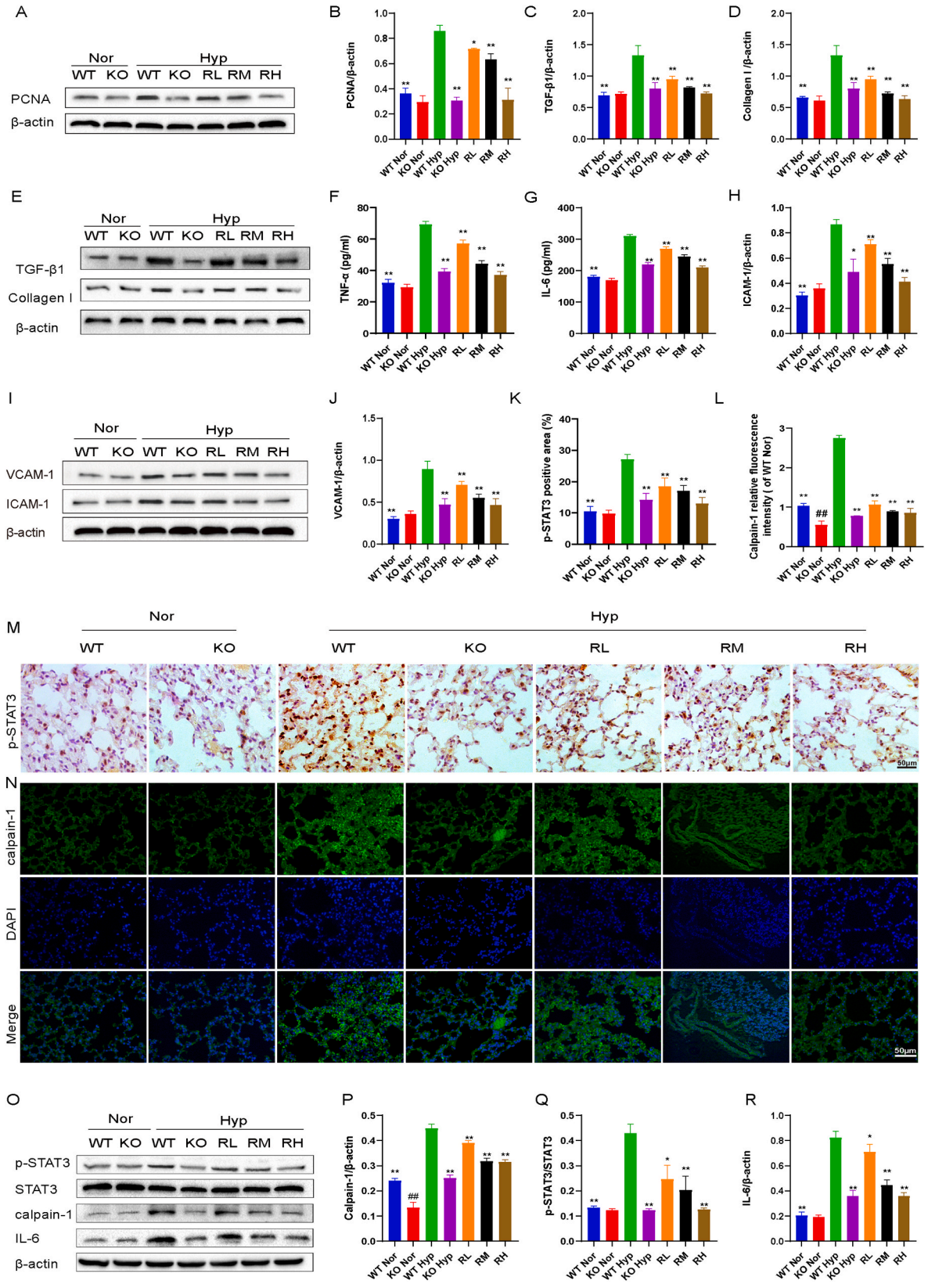

Fig. 3. Ginsenoside Rg1 and Calpain-1 Knockout improve hypoxia-induced inflammatory response and activate calpain-1/STAT3 signaling pathway in HPH mice. (A) (B) Western blot analysis of lung tissue PCNA protein levels (n = 3). (C–E) Western blot analysis of lung tissue TGF-β1 and collagen I protein levels (n = 3). (F) (G) Elisa kit for the determination of TNF-α and IL-6 in mouse serum(n = 8). (H–J) Western blot detection of protein expressed in VCAM-1 and ICAM-1 (n = 3). (K) (M) The expression of p-STAT3 was ascertained by immunohistochemical staining (n = 3). (L) (N) Calpain-1 expression was assessed by immunofluorescence staining (n = 3). (O–R) Western blot assays for expression of calpain-1, p-STAT3 and IL-6 (n = 3). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with WT Hyp group, $^{\#\#}$P < 0.01 compared with WT Nor group).

ratio) of mice in the WT Hyp group was significantly increased, while the W/D ratio of mice in the Rg1 treatment group and KO Hyp group was obviously decreased. These results suggested that Rg1 treatment and Capn1 knockout reduce pulmonary fibrosis in HPH mice.

3.4. Effects of ginsenoside Rg1 and Capn1 knockout on inflammation in HPH mice

Inflammation is vital to the progression of HPH. We continued to analyze the influence of Rg1 and Capn1 knockout on inflammation in HPH. Our data indicated that hypoxia leads to increased serum levels of IL-6 and TNF-α, and Western blot results showed increased protein expression of VCAM-1 and ICAM-1. Rg1 treatment and Capn1 knockdown significantly reduced the concentrations of IL-6 and TNF-α (Fig. 3F and G) and the expression of VCAM-1 and ICAM-1 (Fig. 3H–J). Therefore, the results suggest that Rg1 administration and Capn1 knockdown suppressed inflammation.

3.5. Effects of ginsenoside Rg1 and Capn1 knockout on calpain-1, STAT3 and p-STAT3 protein expression in HPH mice

To explore the influence of Rg1 and calpain-1 knockdown on activation of calpain-1/STAT3 pathway, our data showed that compared with the WT Nor group, the protein expression level of calpain-1 in the lung tissue of the calpain-1 knockout group was reduced. Thus, calpain-1 knockout was successful (Fig. 3 N, O). Hypoxia resulted in increased levels of calpain-1 and increased phosphorylation levels of STAT3. In contrast, Rg1 administration and Capn1 knockdown reduced the expression of calpain-1, IL-6 and p-STAT3 (Fig. 3K–R).

3.6. Effects of ginsenoside Rg1, MDL-28170 and HY-13818 on hypoxia-induced PASMC proliferation

We further explored the effects of Rg1 and calpain-1 inhibition on proliferation at the cellular level. Identification of primary PASMCs by α-SMA immunofluorescence (Fig. 4A). Primary PASMCs were treated with increasing concentrations of Rg1 (0, 10, 20, 40, 80) for 24 h under hypoxic conditions. The CCK8 results showed that Rg1 inhibited the proliferation of PASMCs in a dose-dependent manner, and cell viability was best at an Rg1 concentration of 20 μM (Fig. 4B). Immunofluorescence results showed increased expression of PCNA in PASMCs under hypoxia. Incubation with Rg1 inhibited the expression of this protein. The effect of Rg1 on PASMC proliferation was similar to that of MDL-28170 and HY-13818 (Fig. 4F–E). In addition, the EdU kit showed that Rg1, MDL-28170, and HY-13818 reduced EdU-positive PASMCs under hypoxic conditions (Fig. 4 C, D). These results indicated that Rg1, MDL-28170, and HY-13818 inhibited PASMC proliferation under hypoxia.

3.7. Effects of ginsenoside Rg1, MDL-28170 and HY-13818 on inflammation and fibrosis in hypoxia-induced PASMCs

To explore the effects of ginsenoside Rg1, MDL-28170 and HY-13818 on inflammation and fibrosis. ELISA and Western blot results showed that TNF-α and IL-6 in PASMC supernatants were increased under hypoxic conditions (Fig. 4L and M), and the expression of ICAM-1, VCAM-1, Collagen I and TGF-β1 was increased. Treatment with ginsenoside Rg1, MDL-28170, and HY-13818 reduced the expression of inflammatory and fibrotic factors (Fig. 4G–K).

3.8. Effects of ginsenoside Rg1, MDL-28170 and HY-13818 administration on calpain-1, STAT3 and p-STAT3 protein expression in PASMCs

To probe the influence of ginsenoside Rg1, MDL-28170 and HY-13818 on the expression of calpain-1, STAT3, p-STAT3 and IL-6 proteins in PASMCs were verified by Western blot and cellular immunofluorescence assays. The data showed that the calpain-1 protein level was higher and the IL-6 protein expression and STAT3 phosphorylation level were increased in PASMCs after hypoxia exposure. Rg1 administration therapy, MDL-28170 and HY-13818 reversed the protein expression levels (Fig. 5A–H).

3.9. Effects of Calpain-1 overexpression on hypoxia-induced PASMC proliferation, inflammation and fibrosis

By introducing pLV-CAPN1 into PASMCs overexpressing calpain-1, we further explored the role of calpain-1 in hypoxia-induced PASMC proliferation, inflammation and fibrosis. After stabilized transfection, we found that the expressed of calpain-1 was significantly higher in pLV-CAPN1 group (Fig. 6A and B). Compared with pLV-NC Hyp group, PASMCs cell viability and protein expression levels of PCNA, IL-6 and TGF-β1 were significantly increased in pLV-CAPN1 Hyp group. Furthermore, the expression levels of calpain-1 and p-STAT3 in PASMCs in the pLV-CAPN1 Hyp group were also increased. Compared with those in the pLV-CAPN1 Hyp group, the expression levels of calpain-1, p-STAT3, PCNA, IL-6 and TGF-β1 in the Rg1+pLV-CAPN1 group were significantly reduced. The above results indicated that Rg1 administration eliminated the influence of calpain-1 overexpression. This suggests that Rg1 may act through calpain-1 (Fig. 6C–I).

4. Discussion

Pulmonary arterial hypertension (PAH) is a life-threatening condition [20], and despite increasing evidence that drug therapy increases the survivability of patients with PAH, there is still no cure. Therefore, screening for new medicines and effective therapeutic goals will enable us to further improve the patient's life span and move toward healing. In this research, we assessed the influence of Rg1 on hypoxia-induced PAH in mice and PASMCs and the underlying mechanisms. Our findings show that Rg1 improves pulmonary hemodynamic function, changes in right ventricular hypertrophy and vascular remodeling, suppresses pathological alterations, improves hypoxia-induced proliferation, inflammation and fibrosis, inhibits the increase in calpain-1 and inhibits p-STAT3 signaling.

The main manifestations of vascular remodeling are proliferation of PASMCs, ECM disturbances, and deposition of collagen fibers [21,22]. Continuous pulmonary vasoconstriction is the primary cause of pulmonary vascular resistance and pulmonary artery pressure increases in PAH patients, and concentric wall thickening of the small and medium pulmonary arteries and lumen occlusion are hallmarks of vascular remodeling [23]. Convincing evidence has shown that PASMC proliferation is a basic characteristic of pulmonary vascular remodeling and is the foundation of PAH occurrence and development [24,25]. A previous study showed that vascular inflammation can promote PASMC proliferation, thus aggravating pulmonary artery reconstruction [26,27]. Consistent with the results of clinical reports of macrophage recruitment in vascular lesions in PH patients [28], we found that IL-6 and TNFα promoted inflammatory factors of the inflammatory response in hypoxic mice in lung tissue and serum. In this research, a model of pulmonary hypertension was established by exposing mice to a hypoxic chamber (10% oxygen) for four weeks. We found that after four weeks of hypoxia in mice, interstitial smooth muscle cells in the pulmonary artery had significantly increased thickness and disorganized structure, elevated mPAP, RVSP and RVHI. Increased expression of α-SMA, a smooth muscle actin that is also a pro-fibrotic factor, suggests that small pulmonary arteries are undergoing myelination and vascular fibrosis. Proliferating cell nuclear antigen (PCNA) is an essential factor in DNA replication, The proliferating cell nuclear antigen, Ki67, is considered to be an ideal indicator for detecting cell proliferation, In this study, increased expression of α-SMA, PCNA, Ki-67, TGF-β1 and promotion of proliferation of PASMCs. Ginsenoside Rg1 can relieve the abnormal increase in

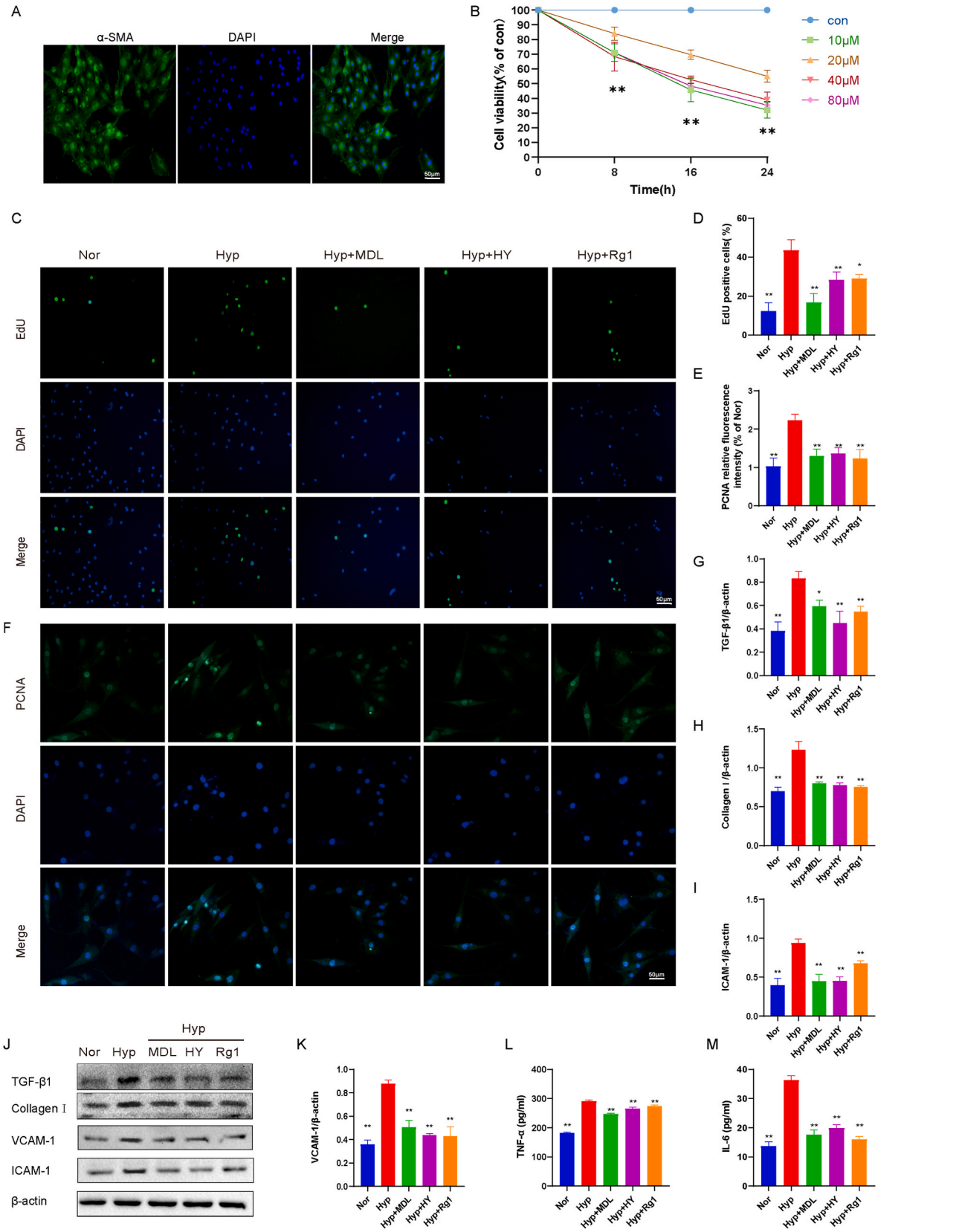

Fig. 4. Ginsenoside Rg1 improves hypoxic-induced PASMCs proliferation, inflammation and fibrosis through the calpain-1/STAT3 signaling pathway. (A)Immunofluorescence staining of α-SMA(n = 3). (B)The CCK-8 detects cell viability at different concentrations(n = 3). (C)(D) The EdU kit detects cell proliferation(n = 5). (E)(F) Using immunofluorescence staining detection of PCNA expression in PASMCs (n = 3). (G–K) The protein level of TGF-β1, Collagen I, VCAM-1 and ICAM-1 in PASMCs was detected by Western blot (n = 3). (L–M) The Elisa kit detects the levels of TNF-α and IL-6 in PASMCs (n = 5). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with Hyp group).

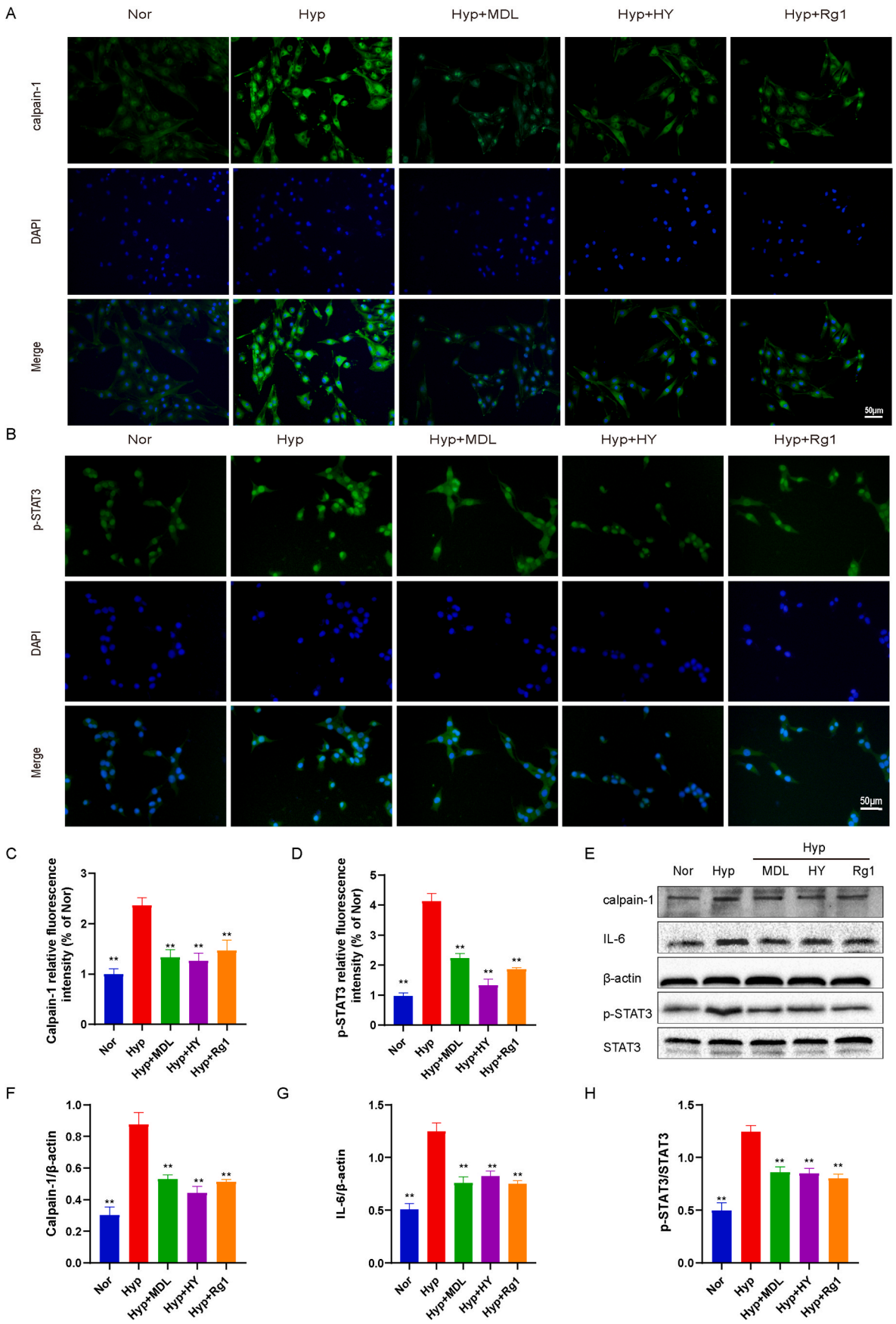

Fig. 5. Effects of ginsenoside Rg1, MDL-28170 and HY-13818 administration on calpain-1, STAT3, p-STAT3 and IL-6 protein expression in PASMCs. (A–D) Immunofluorescence staining of calpain-1 and STAT3 in PASMCs (n = 3). (E–H) The expression of calpain-1, p-STAT3 and IL-6 in PASMCs was detected by Western blot (n = 3). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with Hyp group).

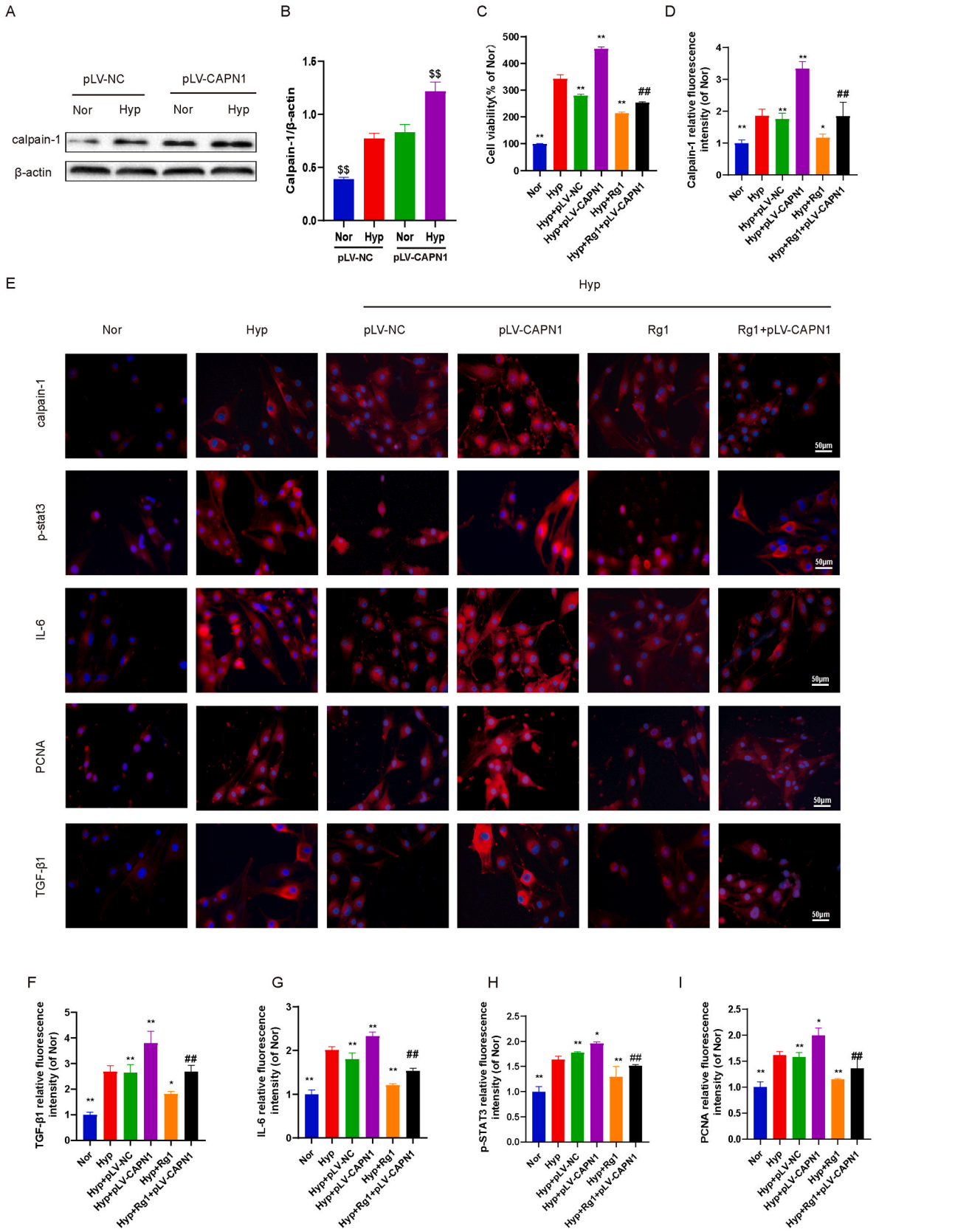

Fig. 6. Effect of calpain-1 overexpression on hypoxic-induced inflammation, fibrosis and proliferation of PASMCs. (A–B) Western blotting assays for calpain-1 levels (n = 3). (C) Cell viability was measured by CCK8(n = 5). (D–I) Immunofluorescence staining in detection of PASMCs calpain-1, p - STAT3, IL - 6, the expression of PCNA and TGF-β1(n = 3). Data are expressed as mean ± SD (*P < 0.05, **P < 0.01 compared with Hyp group, $^{\#\#}$P < 0.01 compared with Hyp + Rg1 group, $^{\$\$}$P < 0.01 compared with Hyp pLV-NC group).

mPAP, RVSP and RVHI. Furthermore, analysis of HE results showed that Rg1 administration reduced hypoxia-induced vessel wall thickening, and ginsenoside Rg1 administration improved the expression of related protein levels with inflammation, proliferation, and fibrosis in lung tissue and PASMCs exposed to low oxygen conditions. These results suggest that Rg1 treatment improves the cell viability of PASMCs after hypoxia, ameliorates in vitro and in vivo hypoxia-induced inflammatory, proliferative, and fibrotic responses and ameliorates hypoxia-induced PAH.

STAT3 is a potential transcription factor that interacts with cell surface peptide receptor-mediated cytokines and growth factors and extracellular signals [29]. STAT3 protein is activated by transcription primarily through tyrosine phosphorylation. Activation of the STAT3 dimer translocates to the nucleus and combines with specific sequences of DNA, leading to target gene transcription [30]. A mounting body of data suggests that STAT3 signaling is activated in the pathogenesis and progression of PAH. In the Monocrotaline (MCT)-induced PAH model, it was found that MCTP targeted pulmonary arteries to induce TGF-β expression by activating the JAK/STAT signaling pathway, and then TGF-β induced IL-6 production through JAK/STAT signaling [31]. STAT3 is an essential transcriptional mediator in inflammatory and hypoxic responses. Mechanistic studies have shown that under hypoxia-induced conditions, JAK2 phosphorylates STAT3, which is subsequently translocated to the cell core and combines with the CCNA2 promoter to regulate the expression of cyclin A2; thus, JAK2 promotes PASMC proliferation and leads to pulmonary vascular remodeling [32]. Our results suggested that hypoxia promoted the expression of p-STAT3, whereas Rg1 downregulated p-STAT3 expression. Furthermore, in PASMCs exposed to hypoxia, HY-13818 treatment downregulated the levels of PCNA, TGF-β1, and IL-6.

Calpain-1 is the main member of the calpain family [33]. Our laboratory have demonstrated that, in addition to regulating hypoxia-induced PAHs, calpain-1 is known to play a crucial role in pulmonary vascular remodeling, mitochondrial dysfunction and brain injury [34–37]. Additionally, the enriched environment promotes functional recovery after stroke by inhibiting calpain-1 activity and subsequently via STAT3-mediated neurogenesis [38]. Consistent with previous studies, our data show that hypoxia promoted the expression of calpain-1, and Rg1 administration therapy downregulated the expression. Additionally, by suppressing calpain-1, the expression of IL-6 was decreased. Furthermore, the phosphorylation of STAT3 to p-STAT3 stimulated by IL-6 is inhibited, and the expression of p-STAT3 is also decreased. To test whether calpain-1 is associated with hypoxia-induced PAH, we used calpain-1 knockdown mice and lentiviral vector transfection of PASMCs. Our results suggest that the deletion of calpain-1 improved inflammation, fibrosis and proliferation in PAH mice and increased p-STAT3 levels. In addition, calpain-1 knockdown and HY-13818 treatment had similar effects to ginsenoside Rg1 treatment. Overexpression of calpain-1 increased the expression level of p-STAT3 in hypoxic PASMCs and promoted the activation of inflammation, proliferation and fibrosis-related protein levels. In animal and cellular experiments, we found that Rg1 inhibits calpain-1 activation in lung tissue and the PASMC hypoxia response. Overall, Rg1 administration increased the viability of PASMCs and decreased the protein expression of calpain-1, p-STAT3, IL-6, TGF-β1 and PCNA. These effects are similar to those of calpain-1 inhibitor and stat inhibitor therapy, and our results further support that Rg1 may alleviate hypoxia-induced PAH vascular remodeling by inhibiting inflammation, proliferation, and fibrosis.

In summary, although the details of the mechanisms have yet to be determined, in this study, our work reveals that ginsenoside Rg1 inhibits the activation of the calpain-1/STAT3 signaling pathway and the onset and progression of pulmonary vascular remodeling, thereby affecting the possible mechanism of hypoxia-induced PAH. The study of the pathogenesis and treatment of HPH provides a new perspective.

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