

Original Article

Salidroside exerts protective effects against chronic hypoxia-induced pulmonary arterial hypertension via AMPK α 1-dependent pathways

Mayun Chen¹, Hui Cai¹, Chang Yu², Peiliang Wu¹, Yangyang Fu¹, Xiaomei Xu¹, Rong Fan¹, Cunlai Xu¹, Yanfan Chen¹, Liangxing Wang¹, Xiaoying Huang¹

¹Division of Pulmonary Medicine, First Affiliated Hospital of Wenzhou Medical University, Key Laboratory of Heart and Lung, Wenzhou, Zhejiang 325000, China; ²Department of Radiology, First Affiliated Hospital of Wenzhou Medical University, Wenzhou, Zhejiang 325000, China

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Abstract: Salidroside, an active ingredient isolated from *Rhodiola rosea*, has shown to exert protective effects against chronic hypoxia-induced pulmonary arterial hypertension (PAH). However, the underlying mechanisms were not well known. Based on our recent reports, we predicted the involvement of adenosine monophosphate-activated protein kinase (AMPK) mediated effects in salidroside regulation of PAH. Firstly, to prove the hypothesis, rats were exposed to chronic hypoxia and treated with increasing concentrations of salidroside or a selective AMPK activator-5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR) for 4 weeks. After salidroside or AICAR treatment, the chronic hypoxia-induced right ventricular hypertrophy and pulmonary artery remodeling were attenuated. Then the effects of salidroside or AICAR on hypoxia-induced excess cellular proliferation and apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs), which contributed to pulmonary arterial remodeling, were investigated. Our results suggested salidroside, as well as AICAR, reversed hypoxia-induced PASMCs proliferation and apoptosis resistance while AMPK inhibitor Compound C enhanced the effects of hypoxia. To reveal the potential cellular mechanisms, activation of AMPK α 1 and expression of the genes related to proliferation and apoptosis were analyzed in PASMCs after salidroside treatment under hypoxia conditions. The results demonstrated salidroside as well as AICAR might inhibit chronic hypoxia-induced PASMCs proliferation via AMPK α 1-P53-P27/P21 pathway and reverse apoptosis resistance via AMPK α 1-P53-Bax/Bcl-2-caspase 9-caspase 3 pathway.

Keywords: Salidroside, pulmonary arterial hypertension, AMPK α 1, apoptosis, proliferation

Introduction

Pulmonary arterial hypertension (PAH) is a devastating disease characterized by increased pulmonary vascular resistance and increased pulmonary arterial pressure, ultimately leading to right ventricular failure and death [1-3]. Active vasoconstriction and vascular remodeling due to the abnormal growth, the excess cellular proliferation, and the apoptosis resistance of pulmonary arterial smooth muscle cells (PASMCs) are the main events in pathology of PAH [4, 5]. Therefore, inhibition of the cell proliferation or induction of the cell apoptosis may be an efficient therapeutic strategy for PAH [6, 7]. Chronic hypoxia-induced PAH is a common

type of PAH, mainly secondary to the disorders of the respiratory systems and links chronic obstructive pulmonary disease (COPD) with cor pulmonale. Hypoxia is also a well-established stimulus to construct animal models of PAH in which hypoxia induces the survival and proliferation of PASMCs [8].

Salidroside is a major bioactive marker substance isolated from *Rhodiola rosea*, which is used to relieve high altitude sickness and acute exacerbation of PAH [9]. A series of evidence has proven that salidroside has many biological properties such as anti-inflammation [10], anti-oxidation [11], anti-stress, anti-cancer, and enhancing immune effects [9, 12, 13]. Recently,

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an *in vitro* study has revealed the inhibitory effects of salidroside on cell proliferation in PSMCs [14]. Furthermore, our previous reports suggested that salidroside can attenuate chronic hypoxia-induced PAH via adenosine receptor 2a (A_{2a}R) related apoptosis pathway [15]. However, knockout of A_{2a}R could not completely block the protective effects of salidroside against PAH (data not shown), suggesting additional molecular mechanisms existing in the cellular process.

Adenosine monophosphate-activated protein kinase (AMPK), a serine/threonine protein kinase, plays a critical role in regulation of energy metabolic homeostasis by switching on catabolic pathways while switching off anabolism [16]. AMPK exists as a heterotrimeric protein with a catalytic α subunit and regulatory β and γ subunits [17]. There are two isoforms of α (α 1 and α 2) and β (β 1 and β 2) and three γ subunits (γ 1, γ 2, and γ 3). AMPK can be activated by allosteric regulation via an increased ratio of adenosine monophosphate (AMP) to adenosine triphosphate (ATP), and its activity is maintained by the inhibition of dephosphorylation through ADP binding and by the phosphorylation of the α subunit (T172) via upstream kinases [18-21]. AMPK is known to play a critical role in cell-regulation, the decision to enter proliferation, autophagy, or apoptosis, and other cell-fate decisions. The activation of AMPK plays an important role in cardiovascular protection [22-24] and a few studies have revealed the involvement of AMPK activation in PAH. Our previous study demonstrates elevated activation of AMPK α 1 by AICAR attenuated chronic hypoxia-induced PAH via inhibiting proliferation and enhancing apoptosis in PSMCs [25]. Igata *et al.* reported that AICAR significantly inhibits proliferation of human aortic SMCs induced by both platelet-derived growth factor-BB (PDGF-BB) and fetal calf serum (FCS) by blocking cell cycle progression through upregulation of P53 and P21 [26]. In addition, salidroside can block progression through G0/G1 to S phase of the cell cycle which is associated with the inhibition of the P27 expression [14]. Considering that both P21 and P27 are downregulators of P53 and P53 can also modulate apoptosis by regulating expressions of Bax and Bcl-2, we predict salidroside might function by an AMPK α 1-P53-P21/P27 axis to inhibit hypoxia-induced PSMCs proliferation and by an AMPK α 1-p53-

Bax/Bcl-2 axis to reverse hypoxia-induced apoptosis resistance, rebalancing the proliferation and apoptosis to inhibit pulmonary arterial remodeling.

In the present study, both *in vivo* and *in vitro* experiments were performed to examine the effects of salidroside on chronic hypoxia-induced PAH and roles of AMPK α 1 pathways in the modulation of cell proliferation and apoptosis of PSMCs.

Materials and methods

Materials

Salidroside, AMPK agonist 5'-aminoimidazole-4-carboxamide ribonucleoside (AICAR), AMPK inhibitor Compound C, collagenase type I, 4', 6-Diamidino-2-phenylindole dihydrochloride (DAPI) were obtained from Sigma (St Louis, MO, USA). Trizol and Sso Advanced SYBR Green Supermix were obtained from Invitrogen (Carlsbad, CA, USA) and Bio-Rad (Hercules, CA, USA), respectively. Fetal bovine serums (FBS), penicillin G, streptomycin, and Dulbecco's Modified Eagle Medium (DMEM, high glucose) were obtained from Gibco BRL (Gaithersburg, MD, USA). Smooth Muscle Cell Growth Medium-2 (SmGM-2) was purchased from Promocell (Gaithersburg, MD, USA). The rabbit antibodies against total AMPK α 1, P53, P27, P21, Bax, Casp 9, β -actin, and α -smooth muscle actin (SMA) were obtained from Abcam (Cambridge, UK). The rabbit antibodies against PCNA, α -actin, GAPDH and phosphorylated AMPK α 1 were purchased from Santa Cruz (CA, USA). The rabbit antibodies against Bcl-2 and Casp 3 were purchased from Cell Signal Technology (CST, MA, USA). Cell counting kit-8 (CCK-8) was purchased from Dojindo Laboratories (Kumamoto, Japan). Alexa fluor 488-labeled goat anti-rabbit IgG (H+L) was purchased from Molecular Probes (Eugene, OR, USA).

Experimental animals and chronic hypoxia model of PAH

Adult male Sprague-Dawley rats (weight 180-220 g) were obtained from the Laboratory Animal Center of Wenzhou Medical College, Wenzhou, Zhejiang, China. Animal housing and experimental protocols were approved by Wenzhou Medical University and all the meth-

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ods were carried out in accordance with the approved guidelines. The rats were randomly assigned to six groups (10 rats per group): normal control group (N), hypoxia group (H), hypoxia plus salidroside groups (2 mg/kg, 8 mg/kg, and 32 mg/kg, named by HS2, HS8, and HS32, respectively), and hypoxia plus AMPK agonist AICAR (1 mg/kg). Intraperitoneal injection was given half an hour before rats were put into hypoxia chamber. The normoxia control group was injected with saline and exposed to room air whereas the hypoxia group was exposed to 8%-11% O₂ (8 hours/day) with the same injection. Salidroside or AICAR were injected at the dose according to the group destination every day. The hypoxia exposure lasts for four weeks.

Measurements of RV hypertrophy

After hypoxia exposure, rats were anesthetized by an intraperitoneal injection of 5% chloral hydrate (400 mg/kg). Two home-made polyethylene (PE) catheters (with inside diameter as 0.9 mm and outside diameter as 1.1 mm), pre-filled with heparin, were connected to the pressure transducers (PowerLab 8/30 multi-channel biological signal recording system, AD Instruments, Colorado Springs, CO, Australia), and inserted into the right ventricle and left pulmonary artery, respectively. Then the mean pulmonary arterial pressure (mPAP) and the mean carotid arterial pressure (mCAP) were recorded. Next the rats were sacrificed by bleeding to death and the hearts were dissected out, divided into right ventricle (RV), left ventricle (LV), and septum (S), washed by PBS, and weighted. The weight ratio of RV to LV plus S was calculated as an index to reflect RV hypertrophy.

Detection of pulmonary artery remodeling

The left lung of every rat was dissected longitudinally at the portapulmonis, fixed in 4% paraformaldehyde overnight, embedded in paraffin, and sectioned at 4 μ m thick. After hematoxylin and eosin (HE) staining and elastic fiber staining, the structure remodeling of the pulmonary arteries were characterized by microscopic evaluation. The pulmonary arteries (external diameters of 50-200 μ m) were chosen randomly and analyzed with Image-Pro Plus, Version 6.0 (Media Cybernetics, USA). The pulmonary artery medial smooth muscle cell layer (PAMT), the percentage of wall areas to total areas (WA/TA%), and the density of nuclei in the medi-

al SMCs were calculated to evaluate pulmonary artery remodeling.

Ultrastructural examination of pulmonary arteries

The lung tissues closed to the lung hilus was sectioned into small pieces (approximately 1 \times 1 \times 3 mm³), fixed with 2.5% glutaraldehyde and 1% osmic acid, dehydrated with acetone, and embedded in epoxy resin 812. And then the fixed tissues were cut into ultrathin sections by ultra-microtome slices V (LKV, Stockholm, Sweden), stained with uranyl acetate and lead citrate, and examined by a Hitachi H-600 transmission electron microscopy (Hitachi, Japan) to evaluate the ultra-structural changes in pulmonary arterioles.

Isolation and cell culture of rat PSMCs

Adult male Sprague-Dawley rats were killed by intraperitoneal injection of 5% chloral hydrate followed by cervical dislocation that was approved by the guideline of Wenzhou Medical University and National Institutes of Health Standards of Animal Care. The heart and lungs were removed and pulmonary vessels were dissected in H-Hank's buffer. After the inner and outer membranes were removed by the aids of anatomy microscope, the pulmonary arterial tissues were digested by 0.2% collagenase type I and incubated for 60-120 min at 37°C. Then the cells were cultured in SmGM-2 medium supplemented with 5% FBS, 2 ng/ml basic fibroblast growth factor, 5 μ g/ml insulin, and 0.5 ng/ml epidermal growth factor maintained at 37°C with 21% O₂ and 5% CO₂. The smooth muscle cell was identified by microscopy and immunofluorescent and immunohistochemistry staining with anti- α -SMA antibody.

Cell proliferation analysis

The effects of salidroside on proliferation of rat PSMCs under hypoxia conditions or not were determined by a cell viability assay using a cell counting kit-8 (CCK-8, Dojindo, Japan). Rat PSMCs were seeded in 96 well plates (1 \times 10⁴ cells/well) and pretreated with increasing concentration of salidroside (0.5, 5, 50, 500 μ mol/L), AICAR (2.0 \times 10³ μ mol/L, or Compound C (40 μ mol/L) before exposure to hypoxia (5% O₂, 5% CO₂, and 90% N₂). After 24 hours of hypoxic exposure, CCK-8 (10 μ l/well) was

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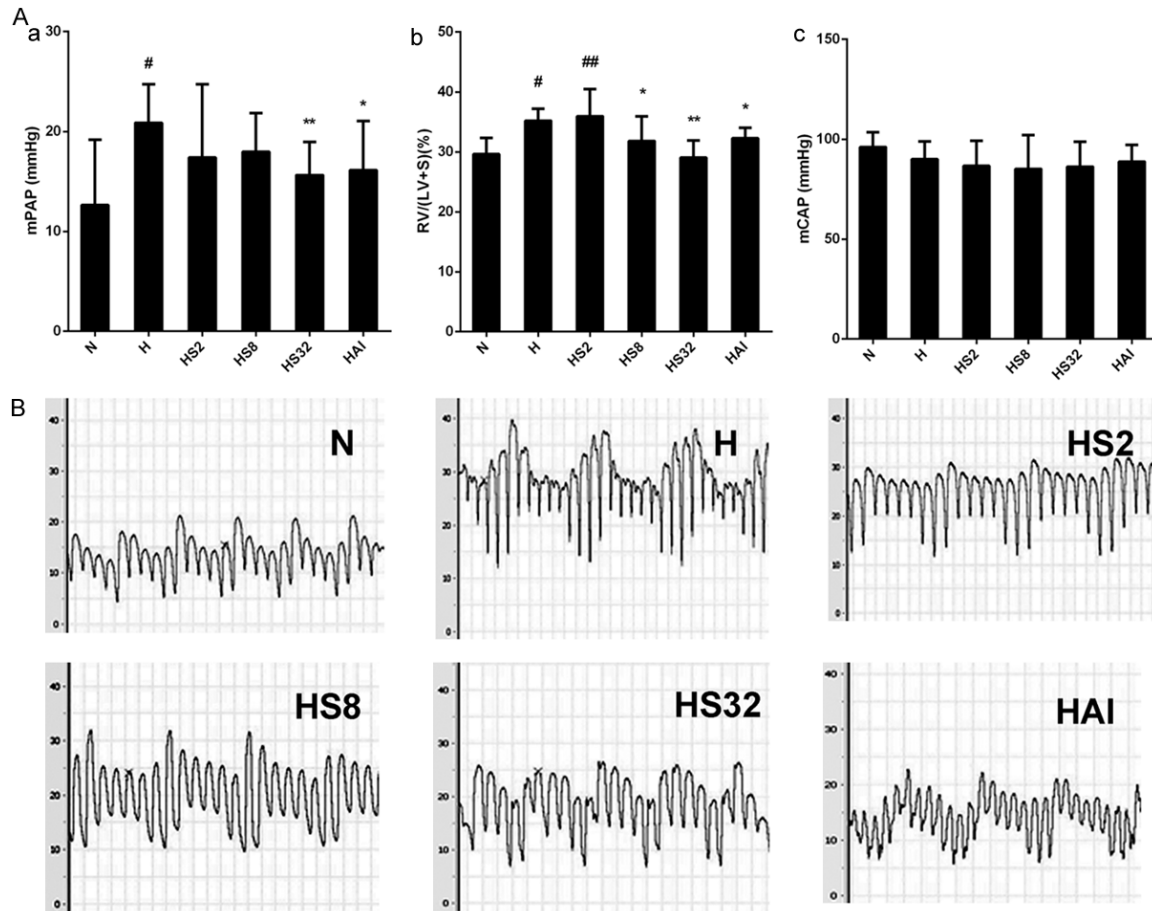


Figure 1. Salidroside inhibited chronic hypoxia-induced PAH in rats. **A.** Rats were exposed to normoxia (N), hypoxia (H), hypoxia with increasing concentrations of salidroside (2 mg/kg, 8 mg/kg, and 32 mg/kg, named as HS2, HS8, and HS32, respectively), or hypoxia with AICAR (1 mg/kg) for 4 weeks. Mean pulmonary arterial pressure (mPAP) and mean carotid arterial pressure (mCAP) were examined. Next, the hearts were dissected out, divided into right ventricle (RV), left ventricle (LV), and septum (S), washed by PBS, and weighted. The weight ratio of RV to LV plus S was calculated as an index to reflect RV hypertrophy. **B.** Representative pictures of PAP waves in the N, H, HS2, HS8, and HS32 groups. [#] $p < 0.05$, ^{##} $p < 0.01$ vs. N group; ^{*} $p < 0.05$, ^{**} $p < 0.01$ vs. the H group; $n = 8$.

added to react with cells for 2 hours. The absorbance was examined at 450 nm by a microplate reader (ELX800, BioTek Instruments, Winooski, VT, USA). IC_{50} was determined according to curvilinear regression between the salidroside concentration and apoptotic index by SPSS 15.0 analysis software. Cell proliferation was also determined by counting the typan blue positive cells.

Immunohistochemistry

After blocking, the paraffin sections were incubated primary polyclonal antibodies against PCNA (1:100), AMPK α 1 (1:100, phosphorylated AMPK α 1 (1:50), α -SMA (1:50). After overnight incubation with primary antibodies, slides were

washed with PBS and incubated with corresponding secondary HRP-linked antibodies (1:100). Lung tissues, incubated with 1% bovine serum albumin (BSA) to replace the specific primary antibody, acted as negative controls. Imaging was assessed by light microscopy and the positive staining was quantified by Image-Pro Plus 6.0 software.

Detection of apoptosis

The apoptosis were qualified using the terminal deoxyribonucleotide transferase-mediated dUTP nick end-labeling (TUNEL) assay (*In Situ* Cell Death Detection Kit, POD) according to the manufacture's instructions. PSMCs cells or tissue sections were fixed with 4% paraformal-

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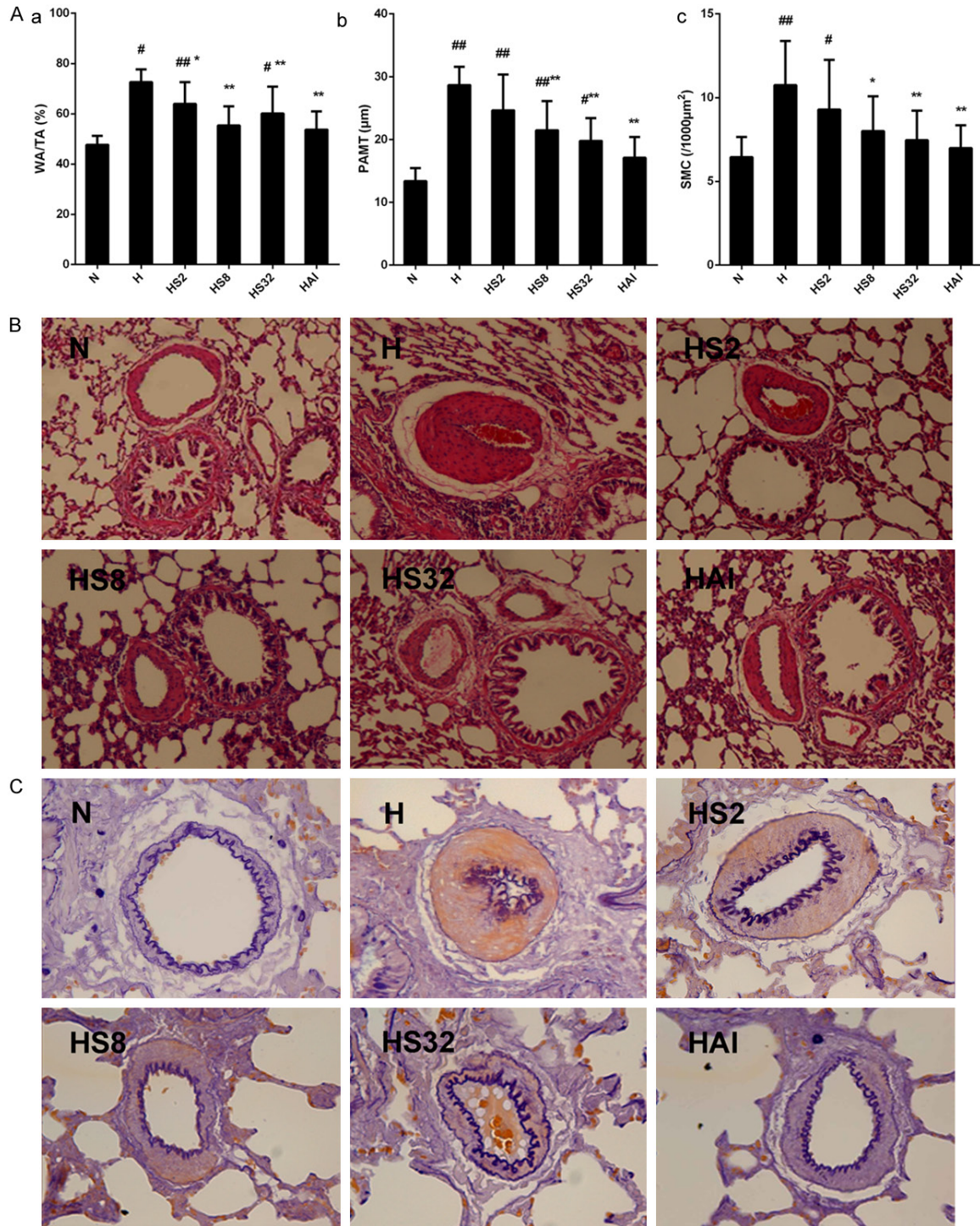


Figure 2. Salidroside improves hypoxia induced pulmonary artery remodeling. (A) Rats were exposed to normoxia (N), hypoxia (H), hypoxia with increasing concentrations of salidroside (2 mg/kg, 8 mg/kg, and 32 mg/kg, named as HS2, HS8, and HS32, respectively), or hypoxia with AICAR (1 mg/kg) for 4 weeks. After hematoxylin and eosin (HE) staining and elastic fiber staining, percentage of wall areas to total areas (WA/TA%), pulmonary artery medial smooth muscle cell layer (PAMT), and the density of nuclei in the medial SMCs were determined. (B, C) Representative photomicrograph of pulmonary artery remodeling analyzed by HE staining (B) ($\times 100$), and elastic fiber staining (C) ($\times 400$). $\#p < 0.05$, $\#\#p < 0.01$ vs. the N group; $p < 0.05$, $**p < 0.01$ vs. the H group; $n = 8$.

dehyde in PBS (pH 7.4) for 1 hour at 25°C followed by subjected to TUNEL assay. Dia-

minobenzidine (DAB) was used as the chromogen and hematoxylin as the counterstain. The

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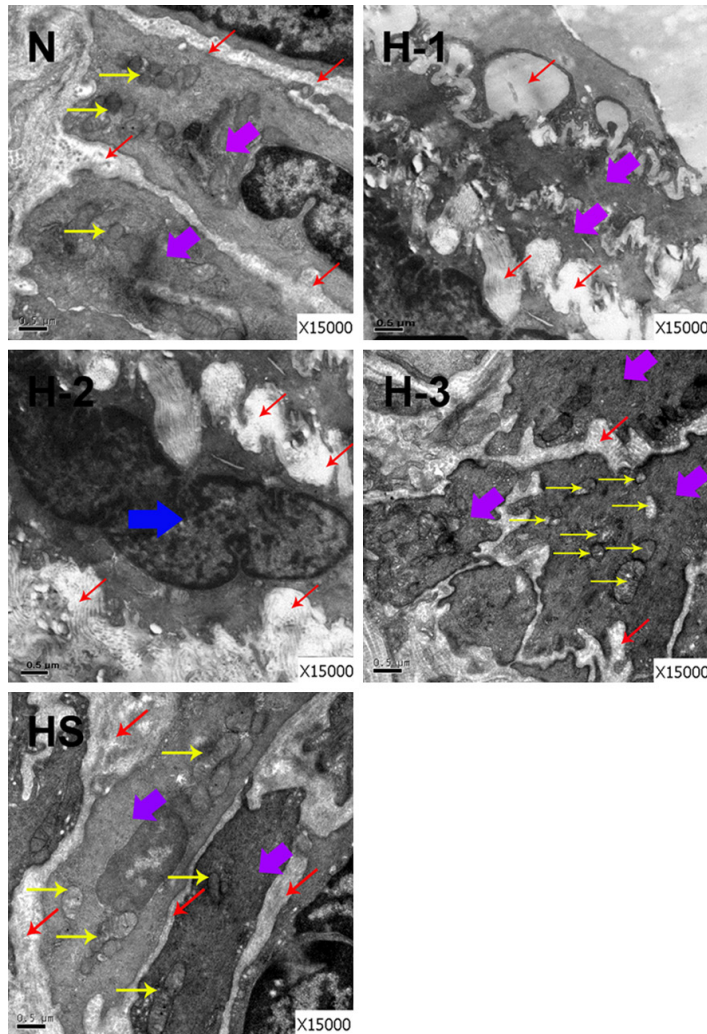


Figure 3. The effect of salidroside on hypoxia induced pulmonary arterial remodeling at ultrastructure level. Rats were exposed to normoxia (N), hypoxia (H), hypoxia with salidroside (32 mg/kg, named as HS) for 4 weeks. The ultrathin sections of lung tissues in N, H, and HS groups were observed under a Hitachi H-600 transmission electron microscopy. Three fields in H group were observed, and named as H-1, H-2, and H-3, respectively. Purple arrow: pulmonary arterial smooth muscle cell (PASMC); Red arrow: collagenous fiber; Yellow arrow: organelle; Blue arrow: muscular vessels smooth muscle cell.

percentage of TUNEL-positive cells was assessed in 5 randomly selected fields in each slide.

Western blotting analysis

Lung tissues with equal weight (50 mg) were homogenized with a glass homogenizer, and lysed in pre-chilled lysis buffer by ultrasonication. PASMCs were lysed with ice-cold RIPA lysis buffer containing PMSF for 30 min. Then the lysates were centrifuged at 12000 rpm for

30 min and 15 min, respectively, at 4°C and the supernatant was collected. The protein concentrations were determined by the Bradford methods. Equal amounts of proteins (20 μ g) were separated with 12% SDS-PAGE, transferred to PVDF membrane, blocked with 5% BSA, and incubated with specific primary antibodies against AMPK α 1 (1:100), phosphorylated AMPK α 1 (1:50), P53 (1:500), P27 (1:500), P21 (1:500), Bax (1:500), Bcl-2 (1:1000), Casp 3 (1:500), Casp 9 (1:500), β -actin (1:1000), and GAPDH (1:50), respectively. Detection of immunoreactive bands was performed using BeyoECL Plus reagents (Beyotime, China). The optical density of immunoblots was calculated with the Quantity one-4.6.2 software (Bio-Rad Laboratories, Hercules, CA, USA).

Statistical analysis

All data were expressed as mean \pm standard deviation (SD). The comparison among more than 3 groups was analyzed by one-way ANOVA, followed by post hoc comparison with LSD test (equal variances assumed) or Dunnett's T3 test (equal variances not assumed). A level of $P < 0.05$ was considered statistically significant. SPSS version 16.0.1 was used to perform all calculations.

Results

Effects of salidroside on chronic hypoxia-induced PAH and pulmonary artery remodeling in rats

To investigate the effects of salidroside on chronic hypoxia-induced PAH, rats were exposed to chronic hypoxia and treated with increasing concentrations of salidroside (2, 8, and 32 mg/kg) or AMPK agonist AICAR (1 mg/kg) for 4 weeks. mPAP, mCAP, and the weight ratio of RV to LV plus S were calculated. As shown in **Figure 1**, mPAP and RV/(LV+S) were increased by hypoxia (H group), demonstrating PAH and RV hypertrophy were successfully

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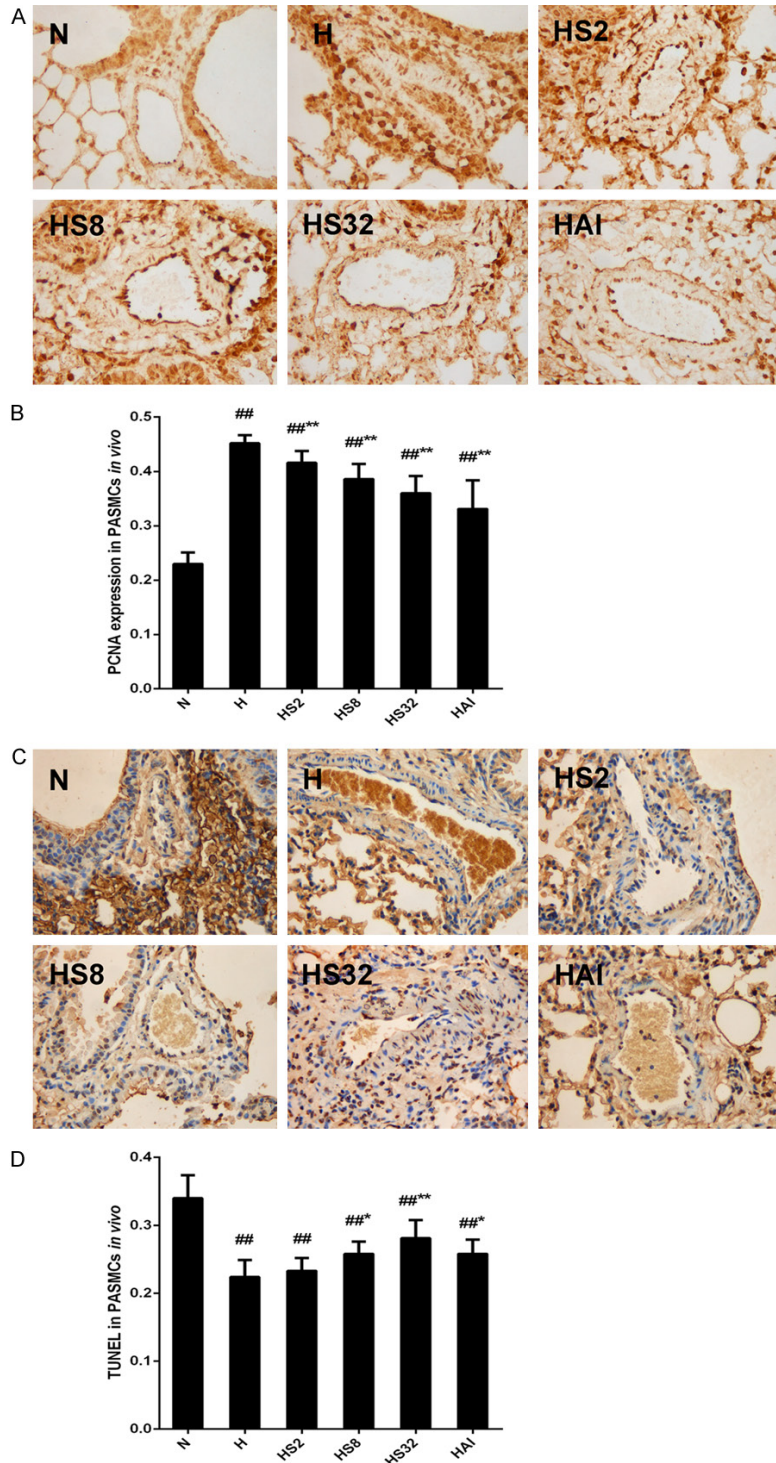


Figure 4. Effects of salidroside and hypoxia on PCNA expression and cell apoptosis in PASMCs in rats. Rats were exposed to normoxia (N), hypoxia (H), hypoxia with increasing concentrations of salidroside (2 mg/kg, 8 mg/kg, and 32 mg/kg, named as HS2, HS8, HS32, respectively), or hypoxia with AICAR (1 mg/kg) for 4 weeks. Lung tissue sections in each group were examined by immunochemical analysis with anti-PCNA antibody (A, B) or TUNEL assay (C, D) ($\times 100$). The PCNA expression and the apoptotic cells were quantified with Image-Pro Plus, Version 6.0. # $p < 0.05$, ## $p < 0.01$ vs. the N group; * $p < 0.05$, ** $p < 0.01$ vs. the H group; $n = 6$.

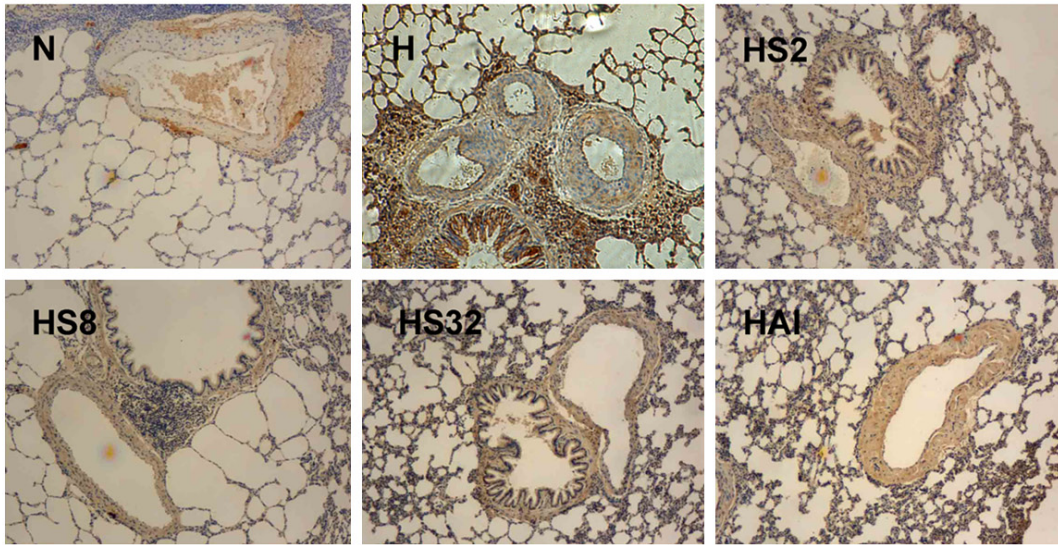
induced by chronic hypoxia. After salidroside treatment, the increase was inhibited and the treatment of 8 mg/kg exerts the strongest inhibitory effects. Similarly, AICAR also inhibited the increase of mPAP and RV/LV+S. The results suggested salidroside could improve hypoxia-induced PAH might via an AMPK axis. In contrast, there was no significant difference in mCAP among all the groups.

Then the effects of salidroside on the pulmonary vascular remodeling, a main characteristic of PAH, was examined. After hematoxylin and eosin (HE) and elastic fiber staining on pulmonary arteries (external diameters of 50-200 μm), the percentage of wall areas to total areas (WA/TA%), the pulmonary artery medial smooth muscle cell layer (PAMT), and the density of nuclei in the medial SMCs were calculated. The prolonged hypoxia exposure resulted in significant increase in WA/TA%, PAMT%, and the density of nuclei in the medial SMCs in H group compared with N group (Figure 2). However, these increases were inhibited by treating with salidroside or AICAR, revealing the pulmonary arterial remodeling induced by chronic hypoxia was attenuated by salidroside and AICAR. In addition, an elevated inflammatory response could be observed in H group after HE staining, and was attenuated by salidroside and AICAR.

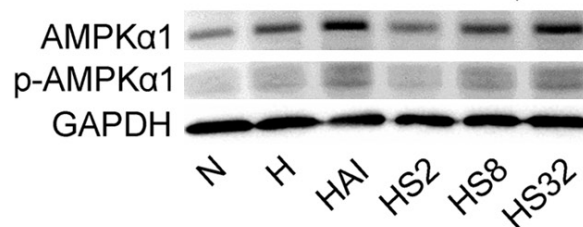
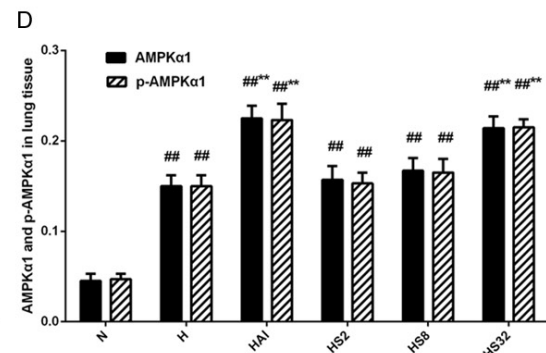
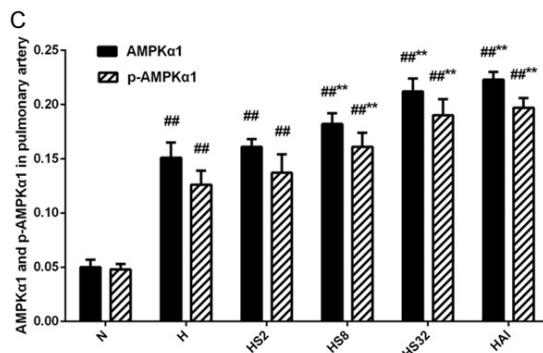
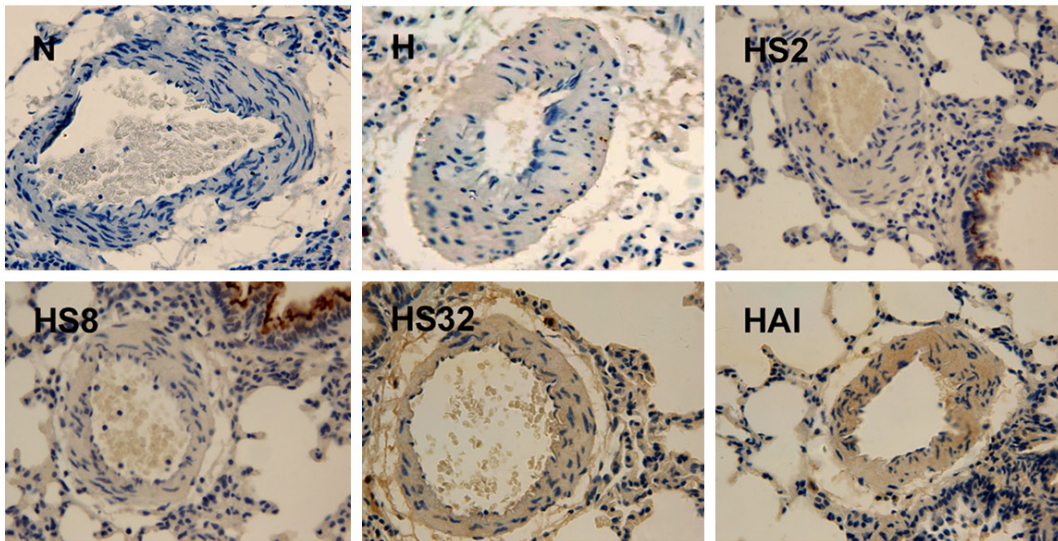
Furthermore, an examination with electron microscope was carried out to verify the

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A **AMPK α 1**



B **p-AMPK α 1**



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Figure 5. Effects of hypoxia and salidroside on AMPK α 1 expression and phosphorylation in PAMSCs in rats. Rats were treated with normoxia (N), hypoxia (H), hypoxia with increasing concentrations of salidroside (HS2, HS8, and HS32), or hypoxia with AMPK agonist AICAR (HA1). AMPK α 1 expression ($\times 100$) and phosphorylation were examined by immunohistochemical staining ($\times 400$) or western blotting # $p < 0.05$, ## $p < 0.01$ vs. the N group; ** $p < 0.01$ vs. the H group; $n = 6$.

inhibitory effects of salidroside on hypoxia-induced pulmonary arterial remodeling at ultra-structural level. In pulmonary arterial smooth muscle cells under normoxia condition, cell membrane and nucleus membrane were smooth, mitochondrial crista was integrated and without vacuolus, and the cells were alignment (**Figure 3**, N). After hypoxia exposure, the shapes of PAMSCs were not consistent, mitochondrial crista was interrupted and full of vacuoles, and the cells were disorganized (**Figure 3**, H1-3). The collagenous fibers were excess proliferated and the internal and external elastic lamina were twisted. There were no immigrated muscular vessels smooth muscle cells in pulmonary artery. Compared with hypoxia group (H), in hypoxia plus salidroside group, PAMSCs were well organized and the abnormal growth of collagenous fiber was reduced obviously (**Figure 3**, HS). And the interrupt of mitochondrial crista and the amount of vacuoles were decreased. The results suggested salidroside attenuated hypoxia induced pulmonary arterial remodeling.

Effects of salidroside on cell proliferation and apoptosis in PAMSCs in vivo

The pulmonary artery remodeling under hypoxia condition is mainly caused by the elevated cell proliferation and impaired cell apoptosis of PAMSCs, which is the major component of the vascular media and the main effectors of the physiological response(s) during pulmonary vascular remodeling [2, 27]. The immunohistochemical staining with anti-PCNA antibody was used to evaluate PAMSCs proliferation in rat. Hypoxia induced an obvious increase of PCNA expression in PAMSCs in rats, indicating an excess proliferation after hypoxia exposure (**Figure 4A** and **4B**). Consistent with the phenotype changes, salidroside or AICAR treatment attenuated the excess proliferation, demonstrating by a decreased expression of PCNA in hypoxia plus salidroside groups and hypoxia plus AICAR groups compared with the N group (**Figure 4A** and **4B**). Next, a TUNEL assay was performed to elucidate the effects of salidroside of hypoxia induced apoptosis resistance of PAMSCs *in vivo*. As shown in **Figure 4C** and **4D**,

an impaired apoptosis was observed in H group, and salidroside reversed the apoptosis resistance in a dose-dependent manner. AICAR treatment also exhibited a promoting effect on PAMSCs apoptosis under hypoxia exposure.

Effects of salidroside on activation of AMPK α 1 in pulmonary artery in hypoxia-PAH rats under hypoxia conditions

The *in vivo* studies suggested salidroside and AICAR, an AMPK agonist, exhibited the similar protective effects against chronic hypoxia-induced PAH, pulmonary artery remodeling, and the excess proliferation and apoptosis-resistance of PAMSCs. Combined with the results in our previous study that activation of AMPK α 1 by AICAR exerted protective effects against PAH [25], we speculated that salidroside might attenuate hypoxia-induced PAH via activating AMPK α 1. Firstly, immunohistochemistry analysis was performed to investigate the expression of total AMPK α 1 and phosphorylated AMPK α 1 in pulmonary arteries (**Figure 5**). Chronic hypoxia led to a remarked increase in total AMPK α 1 and phosphorylated AMPK α 1. Salidroside elevated a higher expression of total and phosphorylated AMPK α 1 in hypoxia plus salidroside groups than that in H group and functioned in a dose-dependent manner. AICAR also elevated a significant increase of total and phosphorylated AMPK α 1 expressions, similar to the high dose of salidroside (32 mg/kg, HS32 group). Secondly, the expressions of total and phosphorylated AMPK α 1 in lung tissues of rats were analyzed by western blotting. Consistent with the immunohistochemical staining results in pulmonary arteries, hypoxia increased significantly both the total and phosphorylated AMPK α 1 expressions, whereas salidroside and AICAR treatments enhanced the increases further (**Figure 5D**).

Effects of salidroside on proliferation and apoptosis of rat PAMSCs under hypoxia conditions in vitro

The *in vivo* studies illustrated salidroside exert protective effects against chronic hypoxia

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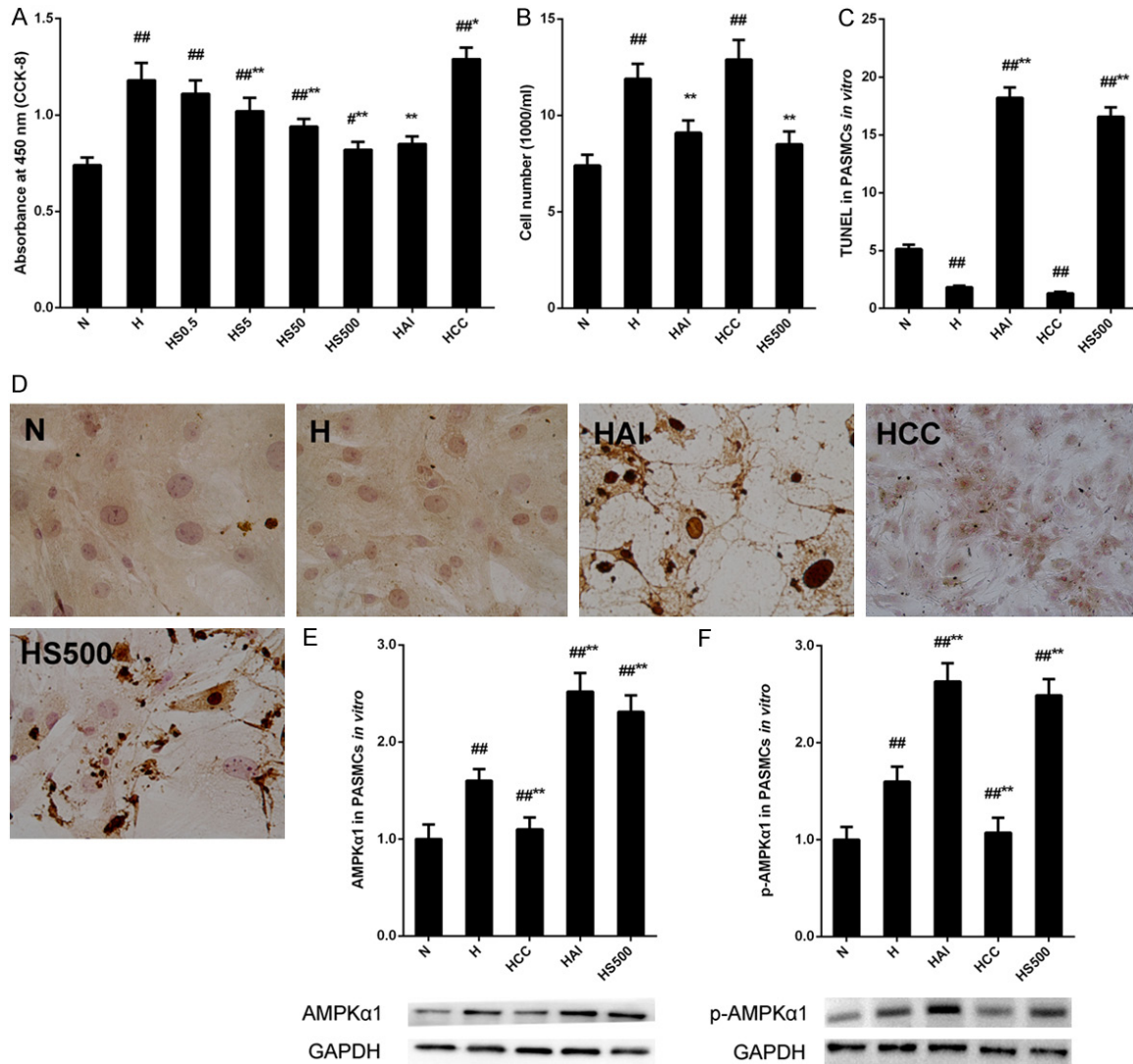


Figure 6. The effects of salidroside and hypoxia on proliferation and apoptosis in PSMCs *in vitro*. (A) Rat PSMCs cells were exposed to hypoxia and treated with increasing concentrations of salidroside (0-500 μ mol/L), AICAR (2.0×10^{-3} μ mol/L) or Compound C (2.0 μ mol/L) and proliferation was analyzed by a CCK-8 assay. (B-D) PSMCs cells were treated with salidroside (500 μ mol/L), AICAR or Compound C under hypoxia conditions. Typan blue staining (B) and TUNEL assay (C, D $\times 100$) were used to analyze cell growth and cell apoptosis of PSMCs. (E, F) AMPK α 1 expression (E) and phosphorylation (F) were examined by western blotting and quantified (C). ### $p < 0.01$ vs. the N group; ** $p < 0.01$ vs. the H group; $n = 6$.

induced PAH via inhibiting cell proliferation and increasing apoptosis of PSMCs, and activating AMPK α 1. Then *in vitro* studies were carried out to verify the effects of salidroside and the mechanisms. PSMCs were firstly isolated from rat and identified (Figure S1). To determine the effects of salidroside on proliferation, rat PSMCs were treated with an increasing concentrations of salidroside (0.5, 5, 50, 500 μ mol/L), AMPK agonist AICAR (2.0×10^3 μ mol/L), or AMPK inhibitor Compound C (40 μ mol/L) under hypoxia conditions for 24 hours. The cell

viability was analyzed by CCK-8 kit. Hypoxia caused a significant increase of cell proliferation and the increase was inhibited by salidroside in a dose-dependent manner and by AICAR while Compound C induced a more excess proliferation than that in hypoxia (H) group (Figure 6A). Meanwhile, cell growth was also evaluated by typan blue staining and the results were consistent with CCK-8 results (Figure 6B). Next, the effects of salidroside and hypoxia on cell apoptosis in rat PSMCs were analyzed with a TUNEL assay (Figure 6C and 6D). After hypoxia

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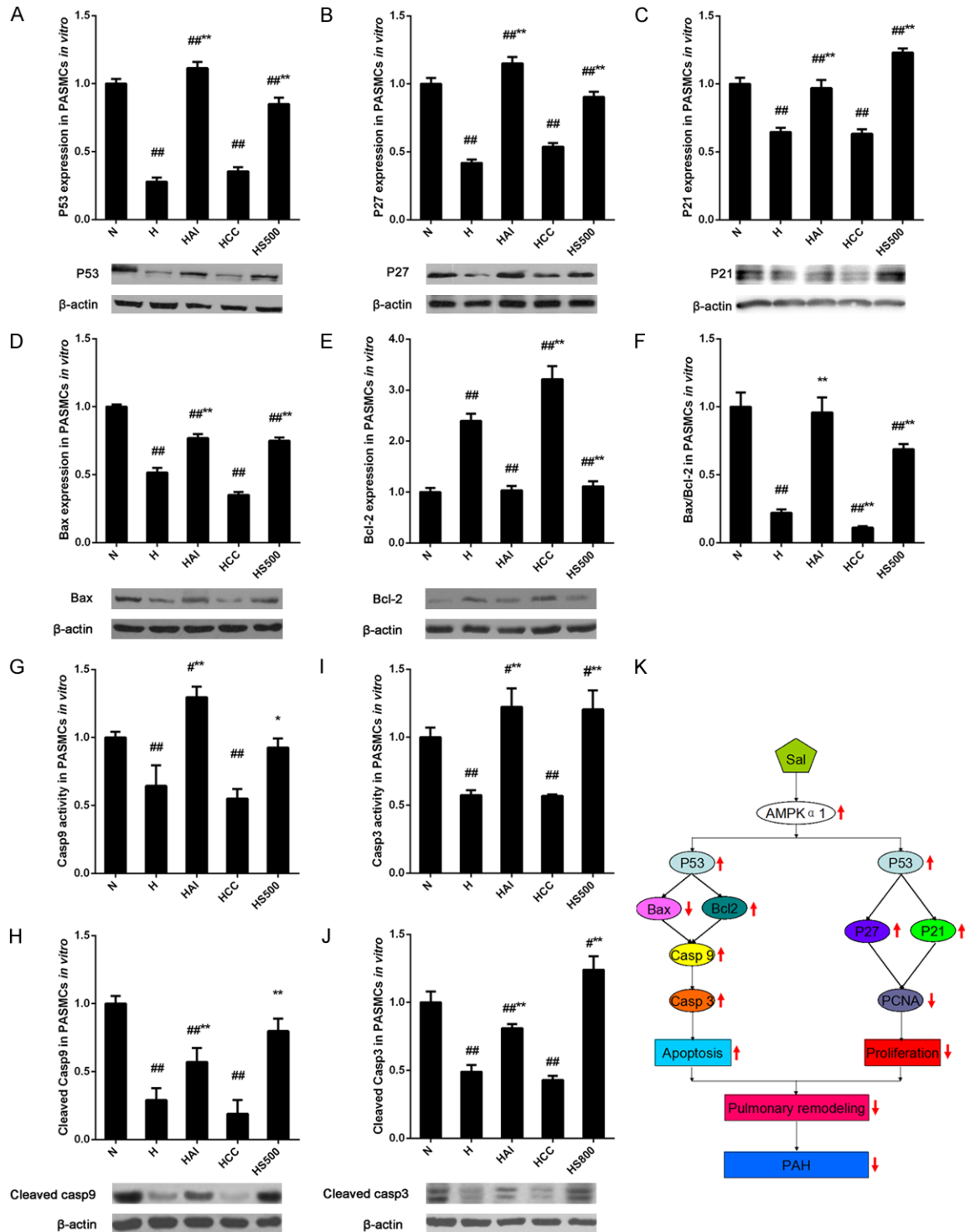


Figure 7. Effects of hypoxia and salidroside on the cell proliferation and apoptosis related genes in PASMCs. (A-E) Rat PASMCs were treated with normoxia (N), hypoxia (H), hypoxia with salidroside (500 μ mol/l, HS500), hypoxia with AMPK agonist AICAR (HAI), or hypoxia with AMPK inhibitor Compound C (HCC) and determined by western blotting with antibodies against P53 (A), P27 (B), P21 (C), Bax (D), and Bcl-2 (E). (F) Protein expression ratio of Bax to Bcl-2. (G-J) The activity and cleavage of caspase 9 and 3 in the cells treated as above were detected. (K) A schematic model illustrating the proposed mechanisms of the protective role of salidroside against hypoxia-induced PAH through AMPK α 1-P53-dependent pathways. ## p <0.01 vs. the N group; ** p <0.01 vs. the H group; n =6.

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exposure, only 1.83% cells were TUNEL positive, about 35% to that in N group. There were 16%~18% cells were positive after salidroside or AICAR treatment under hypoxia conditions which was more than 3-fold to that in N group. The treatment of Compound C combined with hypoxia inhibited the apoptosis, even below than that in H group. Finally, the expressions of total and phosphorylated AMPK α 1 were examined by western blotting in rat PAMSCs *in vitro*, which were treated with salidroside, AICAR, or Compound C under hypoxia conditions. A marked increase of total and phosphorylated AMPK α 1 was observed in H group compared with N group (**Figure 6E**). Salidroside and AICAR upregulated higher expressions compared with H group while Compound C inhibited hypoxia induced total and phosphorylated AMPK α 1 expressions.

Effects of salidroside on the genes related to proliferation and apoptosis in PAMSCs under hypoxia conditions in vitro

The *in vivo* and *in vitro* studies revealed the critical role of AMPK α 1 in the effects of salidroside on cell proliferation and apoptosis in PAH, however, the detailed mechanisms were unclear. The previous studies indicated that activation of AMPK α 1 could inhibit cell proliferation via P53-independent pathway [14, 26]. Then the expressions of P53 and its downstream effectors, P27 and P21, were detected by western blotting after that the PAMSCs were treated with salidroside, AICAR, Compound C under hypoxia conditions. All the protein expressions were downregulated by hypoxia alone or hypoxia with compound C that were reversed by AICAR or salidroside, revealing an AMPK α 1-P53-P27/P21 axis in regulating of cell proliferation (**Figure 7A-C**). The cell apoptosis might also be triggered by a P53-dependent manner, thus apoptosis related proteins were examined. After treatments, the pro-apoptotic factor, Bax, was decreased by hypoxia or hypoxia combined with compound C and increased by AICAR or salidroside under hypoxia conditions, whereas the anti-apoptotic factor, Bcl-2, exert a opposite trend (**Figure 7D-F**). The activity and cleavage of caspase 9 and 3 were further analyzed. Their activity and cleavage were reduced by hypoxia alone or hypoxia combined with compound C and elevated by salidroside or AICAR after hypoxia

treatment (**Figure 7G-J**). These results suggested that salidroside might reverse hypoxia-induced apoptosis resistance via an AMPK α 1-P53-Bax/Bcl-2-caspase 9-caspase 3 pathway.

Discussion

In the previous study, we have revealed the protective effects of salidroside against chronic hypoxia-induced PAH with a mice model and unveiled the important role of A_{2a}R related mitochondria-dependent apoptotic pathway. Unfortunately, other molecular mechanisms were not investigated. The present research confirmed the effects of salidroside with a chronic hypoxia-induced PAH model of rat and provided insight on physiological significance of AMPK activation, by which salidroside attenuated hypoxia-induced PAH. Furthermore, to explore the underlying mechanisms of AMPK activation, we examined the effects of AMPK activation on the cell proliferation and apoptosis related genes and the results suggested that salidroside rebalanced the cell proliferation and apoptosis to improve chronic hypoxia-induced PAH via AMPK α 1-P53 dependent pathways.

PAH is debilitating disease, leads ultimately to right ventricular failure and death, and characterized by increased pulmonary vascular resistance and increased pulmonary arterial pressure [1-3]. Pulmonary arterial remodeling is the main event in pathogenesis of PAH, which is mostly due to the abnormal growth, excess proliferation, and apoptosis resistance in PAMSCs [4, 28, 29]. Therefore, inhibition of the cell proliferation or induction of the cell apoptosis may be an efficient therapeutic strategy for PAH. Salidroside, a pharmacologically active ingredient purified from *Rhodiola* which is used to relieve high altitude sickness and acute exacerbation of PAH, has been revealed to possess multiple pharmacological activities including antiproliferative effects in PAMSCs [9, 14]. Chen *et al.* reported that salidroside significantly inhibits the proliferation and DNA synthesis of PAMSCs induced by platelet-derived growth factor (PDGF)-BB in a dose- and time-dependent manner without cell cytotoxicity [14]. Both in our recent and present studies, we uncovered that salidroside could inhibit chronic hypoxia induced PAH. And the results of immu-

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nohistochemical analysis with anti-PCNA antibody and TUNEL assay demonstrated that salidroside inhibited cell proliferation and reversed apoptosis resistance in PASMCs under hypoxia conditions in rats. The *in vitro* results of CCK-8 assay, typan blue staining, and TUNEL assay also clarified the conclusions.

AMPK is a critical regulator of energy metabolic homeostasis at the cellular whole organism levels that has been highly conserved during evolution [16] and play a critical role in preventing cardiovascular disease including heart disease [30], atherogenesis [31], neointima formation [32, 33], and hypertension [34, 35]. AMPK activation has been shown to reduce myocardial ischemic injury [36, 37], inhibit myocardial hypertrophy [17, 38, 39], and impede the transition from cardiac hypertrophy to heart failure [30]. Furthermore, AMPK has been revealed to inhibit cardiac hypertrophy by promoting autophagy [40]. Activation of AMPK by metformin also inhibit development of PAH by suppressing vascular remodeling in animal model [41] and inhibited PASMCs proliferation *in vitro* [42]. Our previous study showed AMPK activated by AICAR can attenuate hypoxia induced PAH and pulmonary arterial remodeling via inhibiting cell proliferation and enhance apoptosis of PASMCs in rats [25]. Based on the reports, we predicted that AMPK activation might be involved in salidroside regulation of chronic hypoxia induced PAH. *In vivo* experiments in our study showed that the inhibitory effects of salidroside on hypoxia induced PAH, pulmonary arterial remodeling, and PASMCs proliferation and apoptosis resistance was similar with AICAR. Consistent with the *in vivo* results, salidroside as well as AICAR inhibited proliferation and reversed apoptosis resistance in PASMCs *in vitro* while Compound C, an AMPK inhibitor, exhibited an opposite effects. These results illustrated that salidroside exert protective effects against chronic hypoxia induced PAH might via AMPK activation. Both catalytic α subunit of AMPK are expressed in VSMCs while AMPK α 1 is the predominant isoform contributing to total AMPK α and its activation in VSMCs [32]. We determined effects of salidroside on AMPK α 1 expression and phosphorylation under hypoxia condition. The results indicated hypoxia upregulated AMPK α 1 expression and phosphorylation, revealing a compensatory upregulation of AMPK α 1 activation responding

to hypoxia. And salidroside as well as AICAR elevated further upregulation of AMPK α 1 expression and phosphorylation after hypoxia exposure while Compound C inhibited it.

AMPK activation and its function have been addressed in smooth muscle cells (SMCs). Igata *et al.* reported that the AMPK activator AICAR significantly inhibits proliferation of human aortic SMCs induced by both platelet-derived growth factor-BB (PDGF-BB) and fetal calf serum (FCS) by blocking cell cycle progression through upregulation of P53 and P21 [26]. Recently, a potent anti-tumor agent, β -lapachone was shown to inhibit FCS- or PDGF-induced proliferation of VSMCs and to reduce neointimal formation in balloon-injured rat carotid arteries through the LKB1-AMPK-P53-P21 pathway [43]. And also *in vitro* studies suggested that salidroside can block progression through G0/G1 to S phase of the cell cycle which is associated with the elevation of the P27 expression [14]. In the present study, we have found that salidroside induced AMPK α 1 activation might inhibit PASMCs proliferation through a P53-P21/P27-PCNA pathway and enhance cell apoptosis via a P53-Bax/Bcl-2-caspase 9-caspase 3 pathway to attenuate hypoxia induced pulmonary arterial remodeling and consequently improve PAH (**Figure 7K**). In addition, AMPK activation can trigger PI3K/Akt signaling to inhibit cell proliferation [44] and blocking the PASMCs proliferation by salidroside is associated with the suppression of the AKT signaling pathway [14]. Then the AMPK-PI3K/Akt axis might also play a critical role in salidroside inhibition of PASMCs proliferation that will be clarified in our future work.

In summary, salidroside exhibited protective effects against chronic hypoxia induced PAH and pulmonary arterial remodeling partially by AMPK activation. The underlying mechanism might be that AMPK α 1 activation induced by salidroside rebalancing PASMCs proliferation and apoptosis via AMPK α 1-P53-P21/P27-PCNA and AMPK α 1-P53-Bax/Bcl-2-caspase 9-caspase 3 pathways under hypoxia conditions. The evidence provided a significant insight of the protective effects of salidroside against PAH and the underlying mechanisms may become a theoretical basis for the use of salidroside in the treatment of clinical hypoxic pulmonary hypertension.

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Disclosure of conflict of interest

None.

Authors' contribution

The experiments were conceived and designed by MYC, LXW, and XYH, and mainly performed by MYC, HC, and CY. The reagents/materials/analysis tools were prepared and provided by PLW, YYF, XMX, and RF. The Figures were prepared by MYC, HC, CLX, and YFC. The data were analyzed by MYC, HC, and XYH. The manuscript was written by MYC, LXW, and XYH. All authors reviewed the manuscript.

Address correspondence to: Drs. Xiaoying Huang and Liangxing Wang, Division of Pulmonary Medicine, First Affiliated Hospital of Wenzhou Medical University, Key Laboratory of Heart and Lung, Wenzhou, Zhejiang 325000, China. Tel: 0086-0577-55578058; Fax: 0086-0577-55578058; E-mail: drhuangxy@163.com (XYH); Tel: 0086-0577-86-689885; Fax: 0086-0577-86689885; E-mail: wzyxywlx@163.com (LXW)

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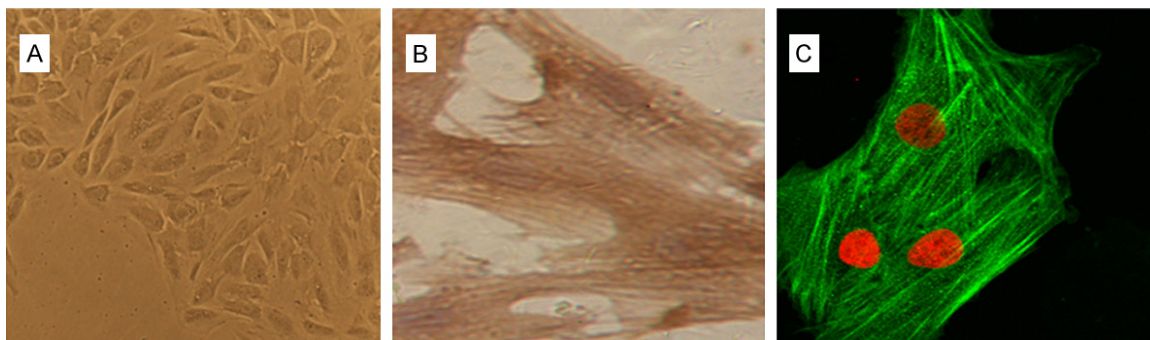


Figure S1. Characterization of PASMCs isolated from rats. (A) Morphology of primary PASMCs cells ($\times 100$). Immunocytochemical staining (B) ($\times 600$) and immunofluorescence staining (C) ($\times 600$) with anti- α -SMA antibody were used to identify PASMCs cells ($\times 400$).