

# Effect of asiaticoside on endothelial cells in hypoxia-induced pulmonary hypertension

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**Abstract.** Pulmonary hypertension (PH) is a chronic progressive disease with limited treatment options. The exact etiology and pathogenesis of PH remain to be elucidated, however there is novel evidence that implicates abnormal endothelial cells (ECs) apoptosis and dysfunction of ECs to be involved in the initiation of PH. Asiaticoside (AS) is a saponin monomer extracted from a medicinal plant called *Centella asiatica*, which had a preventing effect of hypoxia-induced pulmonary hypertension (hypoxic PH) by blocking transforming growth factor- $\beta$ 1/SMAD family member 2/3 signaling in our previous study. The present study demonstrated that AS can prevent the development of hypoxic PH and reverse the established hypoxic PH. AS may activate the nitric oxide (NO)-mediated signals by enhancing the phosphorylation of serine/threonine-specific protein kinase/eNOS, thus promoting NO production, and prevent ECs from hypoxia-induced apoptosis. All these findings imply that AS may be a potential therapeutic option for hypoxic PH patients due to its effect on the vitality and function of endothelial cells.

## Introduction

Pulmonary hypertension (PH) is a chronic progressive disease characterized by increased pulmonary vascular resistance and pulmonary vasculature remodeling. Although PH has a high incidence of mortality (1), the exact etiology and pathogenesis remain to be elucidated. There is increasing evidence that implicates abnormal endothelial cells (ECs) apoptosis and ECs dysfunction to be involved in the initiation and development of PH (2,3). ECs isolated from the idiopathic pulmonary arterial hypertension patients had disordered growth features (4). However, enhanced EC growth and survival, associated with reduced EC apoptosis may prevent the development of PH induced by monocrotaline (5,6). Additionally, dysfunction of ECs may be a consistent marker of PH in rodents and humans (4). In hypoxic PH, the dysfunction of ECs was believed to lead to the reduction in the endothelium-derived nitric oxide (NO) production (7).

NO, working as a potent vasodilator has become an important therapeutic target for hypoxic PH. Endogenous NO is produced by nitric oxide synthase (NOS), which has been efficiently phosphorylated by serine/threonine-specific protein kinase (Akt) activation (8,9). However, the expression of endothelial NOS (eNOS) is reduced (10), whilst the production of endogenous NOS inhibitors, such as asymmetrical dimethylarginine (ADMA) and symmetrical dimethylarginine (SDMA) is enhanced in PH (11). These findings indicated that protecting EC from abnormal apoptosis and normalization of the dysregulated NO-mediated signal in ECs may be potential therapeutic strategies in patients with hypoxic PH.

Asiaticoside (AS) is a saponin monomer extracted from a medicinal plant termed *Centella asiatica*. It has been previously documented to have multiple biological effects, such as anti-oxidant (12), anti-inflammatory (13), anti-hepatofibrotic (14) and acting as a neuroprotector against transient cerebral ischemia and reperfusion (15). However, further investigation that highlights its protective effects on ECs is required. Our previous study revealed rudimentary understanding, that AS may prevent the development of PH by attenuating pulmonary cardiovascular remodeling in hypoxia-induced PAH rats, which may be mediated by blocking the hypoxia-induced

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over activity of transforming growth factor (TGF)- $\beta$ 1/SMAD family member (SMAD) 2/3 signaling (16). However, whether AS may attenuate established hypoxic PH and its effects on the vitality and function of ECs remains to be determined.

Therefore, the present study compared the effect of AS on hypoxic PH rats with different treatment strategies. Subsequently, the effect of AS on EC function was examined by evaluation of NO production and AKT/eNOS activation *in vivo* and *in vitro* and investigated its effects on EC survival and apoptosis *in vitro*. The present study indicated that AS may prevent the development of hypoxic PH and attenuate established hypoxic PH, which may be primarily due to the enhanced NO-mediated signal and the reduced apoptosis of EC under hypoxia.

## Materials and methods

**Animal experimental protocols.** Animal experiments were approved by the Institutional Animal Ethics Committee for Experimentation on Animals of Wenzhou Medical University (Wenzhou, China) and followed the National Institutes of Health Guide for the Care and Use of Laboratory Animals. A total of 40 Adult male Sprague-Dawley rats with a body weight of 180–200 g (SLAC Laboratory Animal Co., Ltd., Shanghai, China) were used in experiments and they were kept at 25°C, 21% O<sub>2</sub>, a 12 h/12 h light/dark cycle and free access to food and water. The rats were then separated randomly into four groups, and each group contained 10 rats: i) Control rats raised in normoxia for 4 weeks (Nox); ii) control rats raised in hypoxia for 4 weeks (Hx); iii) rats raised in hypoxia and received AS (diluted with normal saline; Sigma-Aldrich; Merck Millipore, Darmstadt, Germany) from the same day for 4 weeks (HP); and iv) rats raised in hypoxia for 2 weeks, then received the 4-week AS treatment, i.e., continued another 2 weeks' treatment after finishing the 4-week hypoxia exposure (HT). Hypoxia-induced PH was developed by keeping rats in a sealed, but ventilated hypoxic chamber (9% O<sub>2</sub>, YPC-160D; Changsha Huaxiao Electronic Technology Co., Ltd., Changsha China) as previously described (17). The treatment groups, HP and HT group received AS (50 mg/kg), while Nox and Hx groups received vehicle (normal saline, 1.5–2 ml) administration as control. AS and vehicle were administered daily through intragastric administration.

**Examination of mean pulmonary artery pressure and right ventricular hypertrophy.** After treatment, rats were weighed and received sodium pentobarbital (Sigma-Aldrich; Merck Millipore) anesthetization by intraperitoneal injection (35 mg/kg). Invasive hemodynamic measurements, including mean pulmonary arterial pressure (mPAP) and mean carotid arterial pressure (mCAP) were examined as described previously (18). Following the measurement, the right ventricular (RV) wall was separated from the left ventricular (LV) wall, and the interventricular septum (S) and weighed. An index of right ventricular hypertrophy (RVH) was calculated by right ventricle to left ventricle plus septum ratio.

**Morphometric analysis of pulmonary artery.** Hematoxylin and eosin (H&E) staining (hematoxylin for 5 min and eosin for 30 sec at 25°C) was performed and the images (x400 magnification) of the lung tissue (5- $\mu$ m-thick sections) and pulmonary

arterioles were captured with a microscope digital camera (BX51 microscope; Olympus Corporation, Tokyo, Japan). The percent of medial wall thickness was determined by Image-Pro Plus 6.0 as previously described (19). A small part of flesh lung tissue was maintained in glutaraldehyde. The 3- $\mu$ m-thick sections of each sample were fixed first with 25% glutaraldehyde 2 h at 4°C, then incubated with 1% osmic acid 1 h at 25°C, stained with uranyl acetate for 1 h and lead citrate for 1 h at 25°C, embedded with ethoxyline resin 24 h at 60°C, and then observed under transmission electron microscope detection (H-600 transmission electron microscope; Hitachi, Ltd., Tokyo, Japan).

**Measurement of endothelin (ET)-1, prostacyclin (PGI<sub>2</sub>), cyclic guanosine monophosphate (cGMP) and NO *in vivo*.** The 1 ml blood samples were taken from each rat's heart after hemodynamic measurements to obtain serum at a volume of 150  $\mu$ l, which was centrifuged at 200  $\times$  g, 8 min at 25°C. The levels of ET-1 in the serum of rats were determined by enzyme-linked immunosorbent assay (ELISA) kit (cat. no. ABP52878; Abcam, Cambridge, UK). PGI<sub>2</sub> concentration was also tested by ELISA kit (cat. no. MBS266717; MyBioSource, Inc., San Diego, CA, USA). cGMP levels in lung tissue was quantified by the cGMP Direct Immunoassay kit (cat. no. 581022-96; Abcam). The level of NO measured by quantitating total nitrate/nitrite using nitric oxide colorimetric assay kit (cat. no. K205-100; BioVision, Inc., Milpitas, CA, USA). All the procedures were completed following to the manufacturer's instructions. Data were quantified using a standard curve of known concentrations. Each sample was evaluated in triplicate.

**Cell treatment and NO production of HPAECs.** According to the results of cell viability, HPAECs were divided into the following four groups: i) Nox, cells were cultured under normoxia (21% O<sub>2</sub>, 5% CO<sub>2</sub>); ii) H0, cells were cultured under hypoxia (5% O<sub>2</sub>, 5% CO<sub>2</sub>); iii) H50, cells were cultured with AS (50  $\mu$ g/ml) under hypoxia; iv) HL, cells were cultured with AS (50  $\mu$ g/ml) and LY294002 (20  $\mu$ mol/l) under hypoxia, 3 wells were used per group. All cells were cultured at 37°C for 24 h. The production of NO in HPAECs was quantified by the same colorimetric assay used for the detection of NO levels in rat serum as aforementioned.

**Apoptosis detection.** After treatment, the cells were detected by the terminal deoxynucleotidyl-transferase-mediated dUTP nick end labeling (TUNEL) assay kit (Roche Applied Science, Penzberg, Germany). A total of four groups were fixed of air-dried cell samples with a freshly prepared fixation solution for 1 h at 25°C, then incubated with blocking solution and permeabilization solution sequentially for 10 min and 2 min at 25°C respectively after rinsing slides with PBS in between. The TUNEL reaction mixture was prepared and 50  $\mu$ l was added on sample and incubate for 60 min at 37°C. Samples at last analyzed in a drop of PBS under a fluorescence microscope (CX21FS1; Olympus) with the wavelength of 550 nm. TUNEL-positive cells were calculated as they represent apoptosis cells. For further confirmation, the activity of caspase-3 was quantified using a Caspase-3/CPP32 Colorimetric Assay kit (BioVision, Inc.). Caspase-3 is a key in apoptosis, as its activity reflects the intensity of apoptosis.

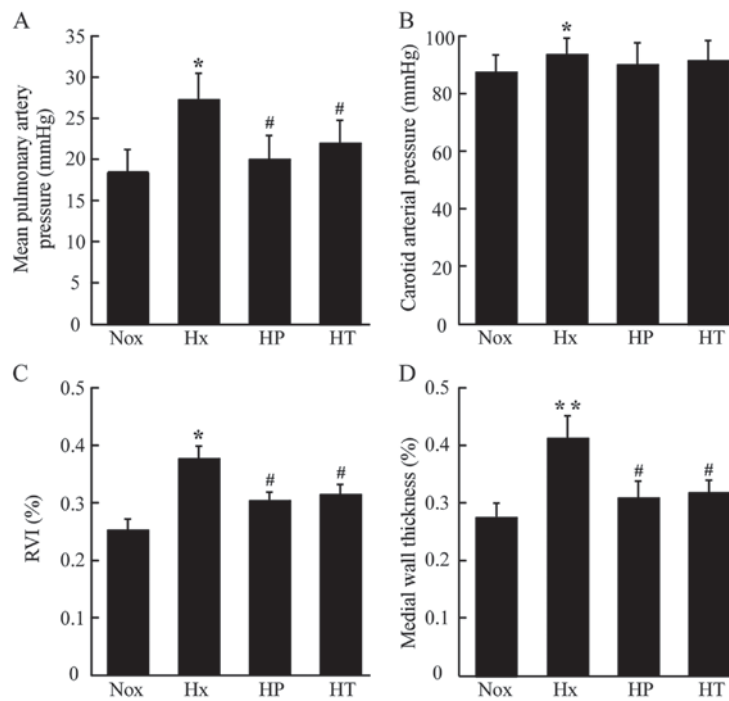


Figure 1. Effect of AS on hypoxia-induced pulmonary hypertension in rats. (A) Mean pulmonary artery pressure. (B) Carotid arterial pressure. (C) Right ventricular hypertrophy (RV/LV+S). (D) Medial wall thickness. Data are presented as the mean  $\pm$  standard deviation. \* $P < 0.05$ , \*\* $P < 0.01$  vs. Nox group; # $P < 0.05$  vs. Hx group,  $n = 10$ . AS, asiaticoside; Nox, control rats raised in normoxia for 4 weeks; Hx, control rats raised in hypoxia for 4 weeks; HP, rats raised in hypoxia and received AS from the same day for 4 weeks; HT, rats raised in hypoxia for 2 weeks, then received the 4-week AS treatment.

Cytosolic extracts were incubated in 96-well plates at 37°C for 1–2 h with DEVD-pNA substrate (200  $\mu$ M final concentration). Absorbance was detected in a microtiter plate reader at 400 nm. Results were calculated by the equation obtained from a standard curve.

**Western blot analysis.** The protein expression of Akt, phosphorylated (p)-Akt at Ser<sup>473</sup>, eNOS, and p-eNOS at Ser<sup>1177</sup> in lung tissue of rats was detected by western blot analysis. Frozen lung tissue was prepared and homogenized in lysis buffer (Beyotime Institute of Biotechnology, Haimen, China), sonicated twice and then centrifuged for 20 min at 10,000  $\times$  g. The expression of these proteins in HPAECs was also quantified 24 h after the aforementioned treatments. Cell proteins were isolated from HPAECs by centrifuging at 4°C for 5 min at 12,000  $\times$  g and using lysis buffer (Beyotime Institute of Biotechnology). To determine the protein concentration of the lysate, the Bradford method was used with bovine serum albumin (ScienCell Research Laboratories, Inc., San Diego, CA, USA) as the standard. Proteins from each sample with equal amounts (25  $\mu$ g) were resolved by SDS-PAGE (12% separation gel) and then transferred onto polyvinylidene fluoride membranes (EMD Millipore, Billerica, MA, USA). Western blots were blocked by incubating with PBS containing 5% skimmed milk for 1 h at room temperature. The membrane was then incubated with the primary antibodies overnight at 4°C. The primary antibodies used were as follows: Anti-eNOS (cat. no. 5880, 1:1,000; Cell Signaling Technology, Inc., Danvers, MA, USA), anti-p-eNOS (cat. no. 9571, 1:1,000; Santa Cruz Biotechnology, Inc., Dallas, TX, USA), anti-Akt (cat. no. 2920, 1:1,000), anti-p-Akt (cat. no. 4051, 1:1,000) (both from Cell Signaling Technology, Inc.), and anti-GAPDH

(cat. no. G8795, 1:1,000; Sigma-Aldrich; Merck Millipore). Subsequently, the blots were incubated with peroxidase conjugated goat anti-rabbit secondary antibody (cat. no. sc-2004, 1:2,000; Santa Cruz Biotechnology, Inc.) for 1 h. Subsequently, peroxidase labeling was visualized via enhanced chemiluminescence reagent provided by Thermo Fisher Scientific, Inc. (Waltham, MA, USA). By scanning the X-ray film (Bio-Rad Laboratories, Inc., Hercules, CA, USA), densitometry results were analyzed using Image-Pro Plus 6.0 (Media Cybernetics, Inc., Rockville, MD, USA) software (National Institutes of Health, Bethesda, MD, USA). All experiments were repeated at least three times.

**Statistical analysis.** Data are expressed as the mean  $\pm$  standard deviation. Between-group mean comparisons were performed using one-way analysis of variance followed by a Student-Newman-Keuls test using R version 3.3.2.  $P < 0.05$  were considered to indicate a statistically significant difference.

## Results

**AS inhibits the development of hypoxic PH, cardiovascular remodeling and endothelial cell injury in hypoxic PH.** As presented in Fig. 1A, mPAP in Hx group increased compared with that in the Nox group. AS administered at 50 mg/kg daily for 4 weeks in prevention and treatment groups (HP group and HT group, respectively) inhibited the elevation of mPAP induced by hypoxia. There was no significant difference between the HP and HT groups. AS seemed to have no impact on the systemic blood pressure, as carotid arterial pressure (CAP), although there was an increased level of CAP in Hx group compared with the Nox group. (Fig. 1B). After



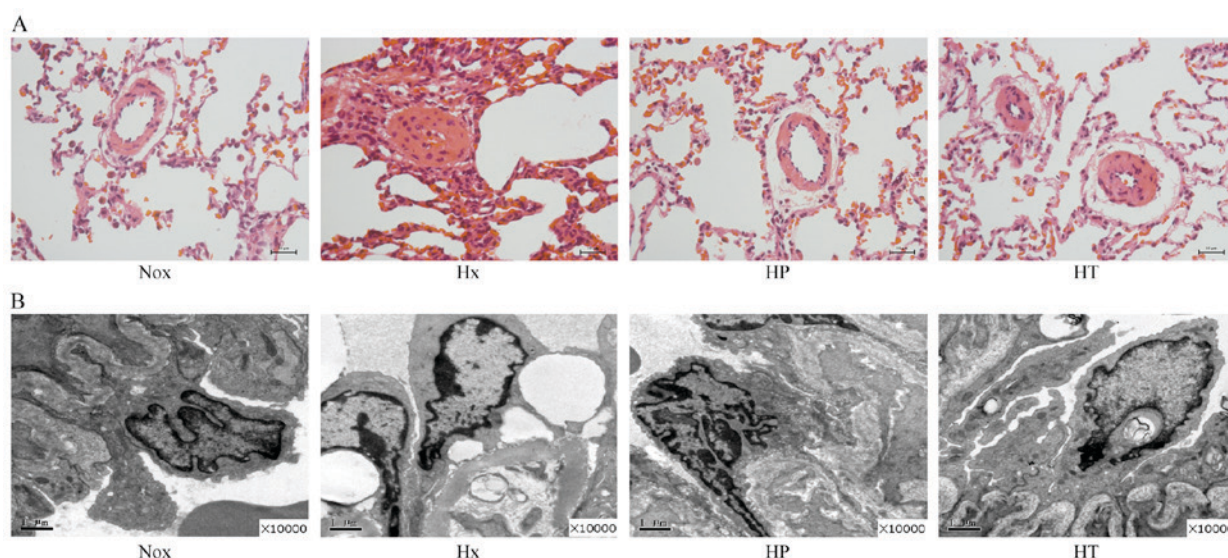


Figure 2. Effect of AS on the morphometry of pulmonary arterioles and epithelial cells in hypoxic pulmonary hypertension rats. (A) Histological features of pulmonary arterioles by hematoxylin and eosin staining. (B) Ultrastructural changes of endothelial cells by transmission electron microscope detection. AS, asiaticoside; Nox, control rats raised in normoxia for 4 weeks; Hx, control rats raised in hypoxia for 4 weeks; HP, rats raised in hypoxia and received AS from the same day for 4 weeks; HT, rats raised in hypoxia for 2 weeks, then received the 4-week AS treatment.

exposure to hypoxia, rats exhibited more severe right ventricular hypertrophy ( $RVI=RV/LV+Sep$ ), which was relieved in both the HP and HT groups (Fig. 1C). Analyses of medial wall thickness of pulmonary arterioles revealed that the Hx group had much higher medial wall thickness than the Nox group, whereas the HT groups showed reduced higher medial wall thickness than Hx (Fig. 1D).

Compared with the Nox group, the lumina of pulmonary arterioles in Hx group was reduced, with inflammatory cells were located along vascular walls (Fig. 2A). These abnormalities were partly ameliorated in the HP and HT groups, with the HP group that started treatment at beginning of hypoxia exposure having reduced pulmonary arteriole wall thickening and inflammatory cell infiltration. At ultrastructure level (Fig. 2B), the pulmonary arteries of the Hx group compared with Nox group showed swelling of endothelial, edema of mitochondria, increased vacuoles, destructive cell junction and exfoliation of basement membrane. Additionally, clustered collagen fibers deposited in adventitia pulmonary arterial walls were observed. Conversely, reduced morphological abnormality of endothelial cells was identified in the HP and HT groups.

*AS modulates dysregulation of ET-1, PGI<sub>2</sub>, cGMP and NO in hypoxic PH.* The circulating concentration of ET-1 was markedly elevated in the Hx group compared with the Nox group, whereas it was lower in the HP and HT groups compared with the in Hx group (Fig. 3A). The serum level of NO which was represented by nitric products was reduced in the Hx group compared with the Nox group. The level of NO was restored in HP group, whereas no significant difference was identified in the HT group (Fig. 3B). The serum levels of PGI<sub>2</sub> showed no significant differences among these groups (Fig. 3C). The cGMP concentrations in lung tissue demonstrated similar pattern as the NO levels in the serum. The HP group had higher cGMP concentration compared with the Hx group, which was significantly reduced compared with Nox group

(Fig. 3D). However, no significant difference was identified between the Hx and HT group.

*AS upregulates activation of Akt and eNOS in lung tissue.* To examine the effect of AS on the activation of Akt and eNOS *in vivo*, the present study assessed the expression of Akt and eNOS, and their phosphorylated products, p-Akt at Ser<sup>473</sup> and p-eNOS at Ser<sup>1177</sup> in lung tissues. The relative ratio of p-Akt/AKT and p-eNOS/eNOS represent the activation of the key proteins in NO-mediated signaling. The phosphorylation of AKT was elevated in the Hx, HP and HT groups. The HP and HT groups had higher levels of activation of AKT compared with the Nox group (Fig. 4A and B). The phosphorylation of eNOS decreased significantly in the Hx group compared with the Nox group. The HP and HT groups were increased in the phosphorylation of eNOS compared with Hx group, whereas the HP group had a higher phosphorylation level compared with the HT group (Fig. 4C and D), which indicated that early treatment of AS contributes to higher phosphorylation levels in hypoxia.

*AS prevents endothelial cells from hypoxia-induced inhibition of cell viability and NO production.* To evaluate whether AS protects HPAECs from hypoxia-induced damage *in vitro*, cell viability was quantified using CCK-8 tests (Fig. 5A). A significant increase in absorbance was observed upon treatment with AS under hypoxia exposure in a dose-dependent manner between 12.5 and 50, and 25 and 50  $\mu\text{g/ml}$  of AS (H25 and H50) compared with control in hypoxia (H0), and their counterparts in normoxia has no significant difference; however, AS (50  $\mu\text{g/ml}$ ) together with LY294002 (20  $\mu\text{mol/l}$ ) significantly suppressed the cell viability either under normoxia or hypoxia condition. As presented in Fig. 5B, NO secretion was reduced in the H0 group compared with the Nox group, whereas it was significantly increased in the H50 group compared with the H0 group. Nevertheless, there was

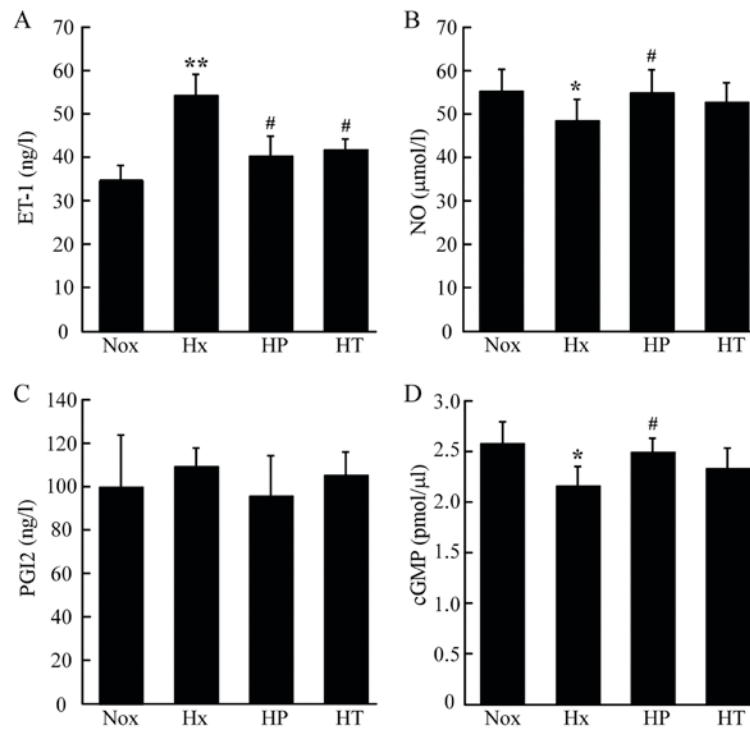


Figure 3. Effect of AS on concentrations of vascular activators in rats tested by ELISA kits. (A) Concentrations of (A) ET-1 (B) NO (C) PGI2 in serum and (D) cGMP in lung tissue. \*P<0.05, \*\*P<0.01 vs. Nox group; #P<0.05 vs. Hx group, n=10. AS, asiaticoside; ET-1, endothelin-1; NO, nitric oxide; PGI2, prostacyclin; cGMP, cyclic guanosine monophosphate; Nox, control rats raised in normoxia for 4 weeks; Hx, control rats raised in hypoxia for 4 weeks; HP, rats raised in hypoxia and received AS from the same day for 4 weeks; HT, rats raised in hypoxia for 2 weeks, then received the 4-week AS treatment.

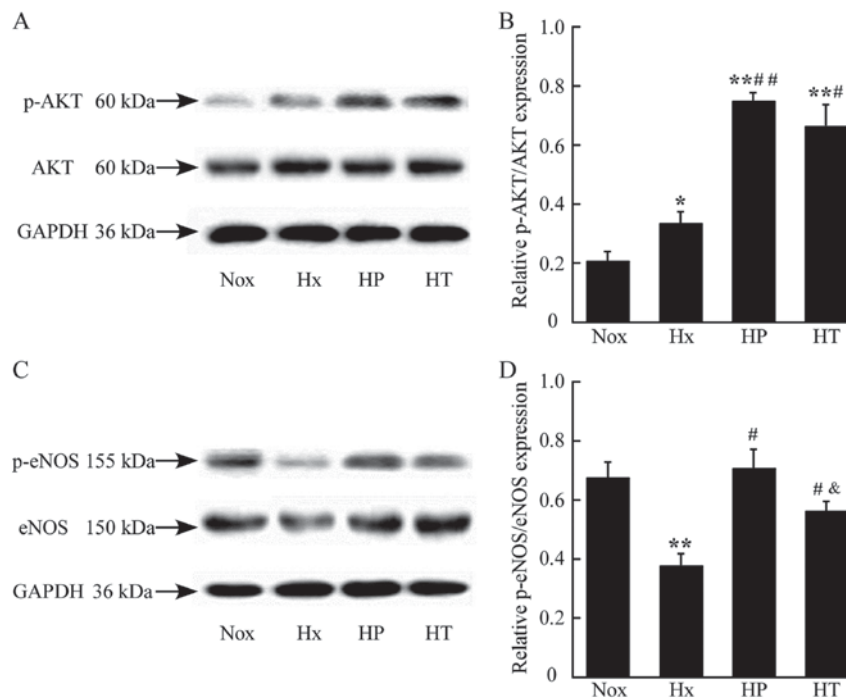


Figure 4. Effect of AS on the activation of eNOS and Akt in lungs of rats assessed by western blotting. (A) Demonstrative immunoblot of Akt and p-Akt in rat lung tissue. (B) Densitometric analysis of expression of p-Akt/Akt ratio. (C) Demonstrative immunoblot of eNOS and p-eNOS in rat lung tissue. (D) Densitometric analysis of expression of p-eNOS/eNOS ratio. \*P<0.05, \*\*P<0.01 vs. Nox group; #P<0.05, ##P<0.01 vs. Hx group; &P<0.05 vs. HP group, n=3. AS, asiaticoside; Akt, serine/threonine-specific protein kinase; p-Akt, phosphorylated-Akt; eNOS, endothelial nitric oxide synthase; Nox, control rats raised in normoxia for 4 weeks; Hx, control rats raised in hypoxia for 4 weeks; HP, rats raised in hypoxia and received AS from the same day for 4 weeks; HT, rats raised in hypoxia for 2 weeks, then received the 4-week AS treatment.

a reduced NO secretion in the HL group compared with the Nox or H50 groups.

AS protects endothelial cells from hypoxia-induced apoptosis. A TUNEL assay was used to determine whether AS

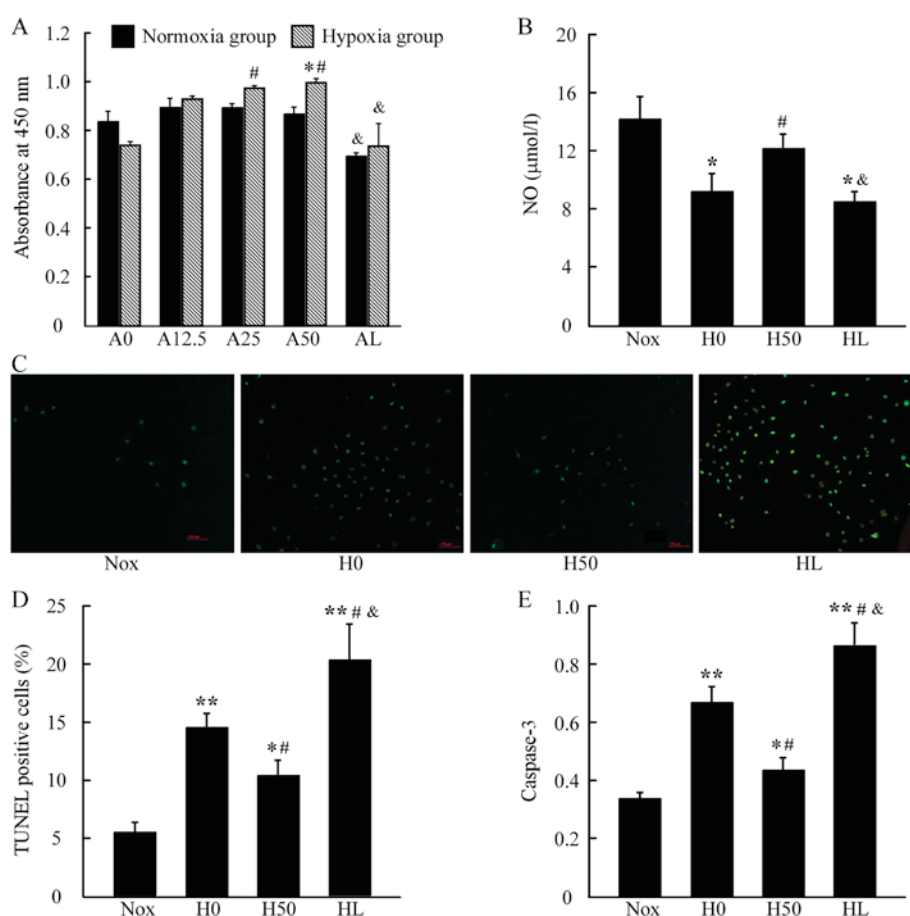


Figure 5. Protective role of AS for HPAECs. (A) Cell viability was determined using Cell Counting Kit-8. (B) NO production in HPAECs. (C and D) TUNEL assay showing the differences of apoptotic rates under specific conditions. (E) Activity of caspase-3 to support the apoptosis evidence. \* $P < 0.05$ , \*\* $P < 0.01$  vs. Nox group; # $P < 0.05$  vs. H0 group; & $P < 0.05$  vs. H50 group,  $n = 3$ . HPAECs, human pulmonary artery endothelial cells; AS, asiaticoside; NO, nitric oxide; Nox, control rats raised in normoxia for 4 weeks.

protects HPAECs by blocking hypoxia-induced apoptosis (Fig. 5C and D). As illustrated, the H0 group exhibited obvious signs of apoptosis compared with Nox, whereas AS treatment (H50 group) prevented hypoxia-induced apoptosis, restoring cell survival under hypoxic conditions. However, the HL group presented a higher level of cell apoptosis compared with the H0 group. Similar pattern was observed in the activity of caspase-3 in these groups. Active caspase-3 was noticeably increased in the H0 group compared to the Nox group, the H50 group had reduced caspase-3 activity compared with the H0 group, whereas there was significantly increased level of caspase-3 activity in the HL group (Fig. 5E).

**AS upregulated and phosphorylation of AKT/eNOS in hypoxia-exposed HPAECs.** Western blot analysis was performed to investigate the effect of the AS treatment on the activation of Akt and eNOS in HPAECs *in vitro*. The hypoxia stimulation of HPAECs induced lower phosphorylation of AKT by detecting the p-AKT/AKT expression ratio, which was significantly increased by AS treatment (50  $\mu\text{g/ml}$ ), whereas treatment combined with LY294002 (HL group) markedly reduced the phosphorylation of AKT induced by the AS treatment. Additionally, AS (50  $\mu\text{g/ml}$ ) upregulated the phosphorylation of eNOS compared with the hypoxia or Nox group ( $P < 0.05$ ), whereas LY294002 and

AS combined (HL group) downregulated the phosphorylation of eNOS ( $P < 0.05$ ). These findings indicated that AS treatment may increase the phosphorylation of AKT/eNOS in hypoxia conditions, whereas LY294002 may inhibit this effect (Fig. 6C and D).

## Discussion

Based on previous observations, the present study determined that in addition to the preventive effect, AS performs a beneficial role in established hypoxic PH as it was evident that AS treatment may restore pulmonary artery pressure without causing systemic hypotension, and attenuate RV hypertrophy, vascular remodeling and ECs morphology changes induced by hypoxia in rats. These findings indicate that AS is a potential option for the prevention and treatment of hypoxic PH.

Endothelial damage is a key initial event in PH. Hypoxia results in the reduction of NO production by interfering the activation of eNOS (20). To the best of our knowledge the present study was the first to demonstrate clearly that AS is capable of maintaining the regular morphology and vital functions of endothelial cells and correcting the NO release *in vivo*. ET-1 is a potent vasoconstrictor, whereas cGMP has vasodilatory and anti-platelet aggregation properties (21). The disequilibrium of those factors underpin various

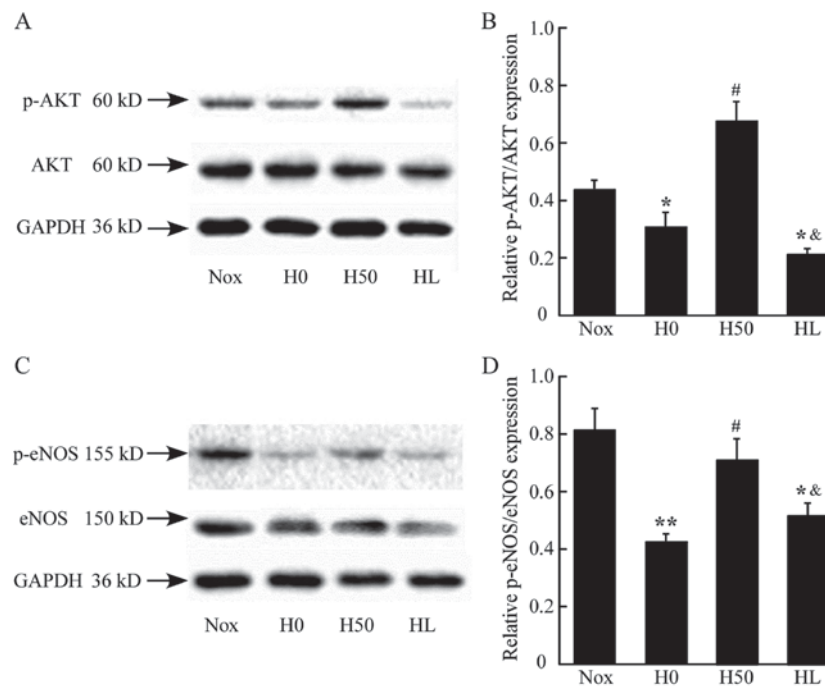


Figure 6. Effect of AS on the activation of eNOS and Akt in HPAECs was assessed by western blotting. (A) Demonstrative immunoblot of Akt and p-Akt in HPAECs. (B) Densitometric analysis of relative expression of p-Akt/Akt ratio. (C) Demonstrative immunoblot of eNOS and p-eNOS in HPAECs. (D) Densitometric analysis of relative expression of p-eNOS/eNOS ratio. \* $P < 0.05$ , \*\* $P < 0.01$  vs. Nox group; # $P < 0.05$  vs. H0 group; & $P < 0.05$  vs. H50 group,  $n = 3$ . AS, asiaticoside; HPAECs, human pulmonary artery endothelial cells; Akt, serine/threonine-specific protein kinase; p-Akt, phosphorylated-Akt; eNOS, endothelial nitric oxide synthase.

morphological and hemodynamic changes in PH (22). In addition to the ultrastructural restoration of ECs, misadjusted levels of vascular mediators like ET-1, cGMP and NO were ameliorated following AS treatment. It is of note, that the AS treatment administered 2 weeks after the hypoxia exposure (HT group) had reduced levels of cGMP and NO restoration *in vivo*, which suggested that early treatment would bring about improved outcomes. Increased NO production by AS was consistent with the significant activated phosphorylation of eNOS at Ser<sup>117</sup>. Akt has been determined to activate the phosphorylation of eNOS, inhibit apoptotic processes, thus interfering with cellular survival pathways (23). The upregulation of Akt and eNOS activation by AS treatment in the present study may contribute to the normalization of NO-mediated signal and the preservation of endothelial cell morphology and function, which inhibits the progression of hypoxic PH in rats.

The present study confirmed the role of AS on the PI3K/Akt signaling pathway by the experiments in HPAECs *in vitro* using a specific PI3K inhibitor, LY294002. The PI3K/Akt signaling pathway has a critical role in cell survival during hypoxia, and phosphorylation of Akt protects cells against hypoxia-induced apoptosis (24,25). The present study determined that hypoxia decreased cell viability and induced apoptosis in HPAECs, which is in accordance with a previous study (26); however, this was significantly reversed by AS (50  $\mu\text{g/ml}$ ) treatment, which demonstrated the protective effects on HPAECs. However, the cytoprotective effect of AS was reduced by PI3K inhibitors LY294002 which induced apoptosis and inhibited viability in ECs under hypoxia. LY294002 may also reduce the effect of AS on the activation of AKT and eNOS, which indicates that AS activated eNOS through a PI3K/Akt-dependent mechanism in

HPAECs, which eventually resulted in the reduced generation of NO by ECs. To the best of our knowledge for the first time, the present study demonstrated that AS treatment upregulated the PI3K/Akt/eNOS pathway *in vivo* and *in vitro*.

There are limitations of the present study. The rat hypoxic PH model used cannot fully represent the molecular complexity present in human patients. Additionally, AS may enhance NO production via activation of the AKT/eNOS pathway, whereas the precise molecular mechanisms underlying the process and their cause-and-effect relationship remain to be elucidated. Additional studies are required in order to resolve these questions.

In conclusion, the present findings suggest that there are significant effects of AS on preventing and reversing hypoxic PH. The present study revealed that the AS acted to promote NO production in circulation of a hypoxic PH rat model, and in HPAECs culture under hypoxia where it had an inhibitory effect on hypoxia-induced apoptosis. The AS-mediated protective effect on ECs was accompanied by phosphorylation of Akt and eNOS *in vivo* and *in vitro*. In addition, this protective effect was significantly inhibited by LY294002 treatment *in vitro*, indicating that PI3K/Akt/eNOS signal pathways have key roles in hypoxic PH and act as a possible target for AS treatment in hypoxic PH. These findings imply that AS may be a potential therapeutic option for hypoxic PH.

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