

Resveratrol inhibits hypoxia-induced proliferation and migration of pulmonary artery vascular smooth muscle cells by inhibiting the phosphoinositide 3-kinase/protein kinase B signaling pathway

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Abstract. Hypoxia is a risk factor for severe chronic obstructive pulmonary disease, which aggravates the disease and may cause mortality by inducing hypoxic pulmonary hypertension (HPH). Proliferation and migration of pulmonary artery smooth muscle cells (PASMCs) may mediate this effect. Resveratrol is a phenolic compound extracted from a plant and has been reported to alleviate HPH, although the underlying mechanisms remained to be elucidated. In cancer, resveratrol has been reported to abrogate the phosphoinositide 3-kinase/protein kinase B (AKT) signaling pathway, thereby inhibiting tumor development. Therefore, the present study aimed to investigate the role of resveratrol in preventing PASMCs from proliferating and migrating. Resveratrol was demonstrated to be inhibitory in a dose-dependent manner on hypoxia-induced cell proliferation and migration, and protein expression levels of phosphorylated AKT and AKT. Additionally, resveratrol was identified to act synergistically with LY-294002, a phosphorylation inhibitor of AKT, but antagonistically with insulin-like growth factor-1, an agonist of AKT phosphorylation. This suggested that resveratrol may reduce proliferation and migration by diminishing expression and phosphorylation of AKT, thereby preventing development of HPH.

Introduction

Chronic obstructive pulmonary disease (COPD) is chronic airway inflammation characterized by persistent airflow

limitation. Pulmonary hypertension is a pathophysiological status which accompanies abnormally increased pulmonary artery pressure, eventually leading to circulatory failure or mortality (1). The majority of patients with severe COPD in intensive care units are in a hypoxic state. Hypoxic environments may cause pulmonary artery vasoconstriction, pulmonary artery smooth muscle cell (PASMC) proliferation and migration, vascular matrix reconstruction and a series of pathophysiological changes, which ultimately leads to the occurrence of hypoxic pulmonary hypertension (HPH) (2). Previous studies have indicated that proliferation and migration of PASMCs serves a critical role in the pathological development process of HPH (3).

Resveratrol is a phenolic compound extracted from a plant, and has significant anti-inflammatory, antioxidant and anti-aging biological effects (4,5). Csiszar *et al* (3) demonstrated that resveratrol may inhibit the development of pulmonary hypertension induced by monocrotaline in rats; however, the underlying mechanisms remain unknown. Previous studies have used resveratrol to examine its anti-tumor effects and its specific roles in abrogating cell proliferation and inducing apoptosis via downregulation of signal transducer and activator of transcription 3 and nuclear factor- κ B (6-10). Protein kinase B (AKT) is a member of serine/threonine protein kinase family and responds to a variety of stimuli, including protein phosphatases, stress and growth factor stimulation. It is additionally implicated in tumorigenesis, and is activated by phosphoinositide 3-kinase (PI3K) (11). Resveratrol has been reported to inhibit AKT activity and induce apoptosis in human uterine cancer cells (12,13). Jiang *et al* (14) previously indicated downregulation of PI3K/AKT/mammalian target of rapamycin (mTOR) signaling pathways in human U251 glioma cells. Similar to its role in tumorigenesis, activation of AKT is important for preventing apoptosis of PASMCs (15), which may be induced by hypoxia (16). Therefore, the present study aimed to investigate the potential antiproliferative effect of resveratrol on PASMCs under hypoxic conditions via downregulation of the PI3K/AKT signaling pathway. The present study investigated the role of resveratrol by examining alterations in expression levels of genes associated with the PI3K/AKT pathways, proliferation and migration, and comparing the viability

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and wound healing rate between treated and control excised rat PAMSCs.

Materials and methods

Experimental animals. Twenty-five Sprague-Dawley rats (age, 50 days; weight, 150-180 g) of specific pathogen free level of either sex were purchased from the Laboratory Animal Center of Zhejiang Medical Academy (Hangzhou, China). Animals were housed with a regular 12 h light/dark cycle at a controlled temperature ($25\pm 2^\circ\text{C}$), with a humidity of 76% and free access to food and water. The current study was reviewed and approved by the Institutional Animal Care and Ethics Committee of The Third Affiliated Hospital of Qiqihar Medical University (Qiqihar, China), and was conducted according to the US National Institutes of Health and the European Commission guidelines.

Experimental reagents. Ether, methanol, 75% and 95% ethanol, anhydrous ethanol and anhydrous methanol were purchased from Hangzhou Chemical Reagent Co., Ltd. (Hangzhou, Zhejiang, China). Dulbecco's modified Eagle's medium (DMEM), PBS, 0.25% trypsin-ethylenediaminetetraacetic acid (EDTA), penicillin and streptomycin were obtained from Hangzhou Genom Biological Pharmaceutical Technology Co., Ltd. (Hangzhou, Zhejiang, China). Resveratrol and homocysteine were purchased from Sigma-Aldrich; Merck KGaA (Darmstadt, Germany); fetal bovine serum (FBS) was obtained from Gibco; Thermo Fisher Scientific, Inc. (Waltham, MA, USA); MTT was purchased from Amresco, LLC (Solon, OH, USA); 4', 6-diamidino-2-phenylindole (DAPI) was obtained from Roche Diagnostics (Basel, Switzerland). The following primary antibodies were purchased from Abcam (Cambridge, UK): Monoclonal rabbit anti-rat SM-actin (cat. no. ab5694; 1:1,000), rabbit anti-rat p21 (cat. no. ab109520; 1:1,000), anti-p27 (cat. no. ab32034; 1:1,000), anti-matrix metalloproteinase (MMP)-2 (cat. no. ab92536; 1:1,000) and polyclonal anti-MMP-9 (cat. no. ab194314; 1:1,000). Rabbit anti-AKT (cat. no. 4691; 1:1,000) and phosphorylated (p)-AKT polyclonal primary antibodies (cat. no. 4060; 1:2,000) were obtained from Cell Signaling Technology, Inc. (Danvers, MA, USA). Horseradish peroxidase (HRP)-conjugated goat anti-rabbit (cat. no. 111035003; 1:15,000) and fluorescein isothiocyanate-labelled goat anti-rabbit IgG (cat. no. 111095003; 1:15,000) secondary antibodies were purchased from Jackson ImmunoResearch Laboratories, Inc. (Baltimore, PA, USA). Western blotting and gelatin zymography relative reagents were obtained from Beyotime Institute of Biotechnology (Haimen, China).

Primary cell culture and identification of rat PAMSCs. Rats were anesthetized and sacrificed by procedures reviewed and approved by the Committee on Animal Resources of Qiqihar Medical University (Qiqihar, China). Anesthesia was produced using atropine (0.05 mg/kg; subcutaneous administration) purchased from Sigma-Aldrich; Merck KGaA. A tissue explants adherent method was used to culture rat PAMSCs; morphological assay and immunocytochemistry were used to detect SM-actin expression to identify PAMSCs, and purity of PAMSCs was evaluated by the association between SM-actin

and DAPI in the nuclei. Cultured rat PAMSCs at passages 3-5 were used for further experiments. Culture media containing 5% FBS and different concentrations (10, 30 and 100 $\mu\text{mol/l}$) of resveratrol (Tianjin Jianfeng Biotechnology Co., Ltd., Tianjin, China) was used in the experiments.

A hypoxic environment for PAMSCs was induced using an autonomous plexiglass chamber supplied with 5% CO_2 and 95% N_2 at 20 ml/min. A CR-2 oxygen detector was applied to monitor the oxygen concentration of the chamber and maintained levels at 3%. Finally, the chamber was incubated at 37°C for 3 days.

PAMSC MTT proliferation assay. PAMSCs at passages 3-5 were digested with 0.25% trypsin, suspended in DMEM supplemented with 10% FBS. Cells were seeded into a 96-well plate at a density of 6×10^3 cells/well. When the cultured cells grew against the wall of the plate, serum-free medium was added for 24 h for cell synchronization. Resveratrol (10, 30 or 100 $\mu\text{mol/l}$), or a combination of resveratrol with LY-294002, a PI3K inhibitor, or insulin-like growth factor-1 (IGF-1), was added to the cells for 24, 48 or 72 h. For the positive control, saline was added to cells in hypoxic conditions. Cells not exposed to hypoxia and treated with saline served as the negative control. Each group had three duplication wells, and two blank wells without cells were reserved. Following culture, 20 μl MTT was added to each culture well and cells were incubated at 37°C for 4-6 h. Subsequently, the supernatant was discarded and 150 μl dimethyl sulfoxide was added to each well for 10 min, with agitation. The optical density (OD) value was measured at a wavelength of 492 nm using an MTT enzyme-linked immunometric meter. The growth curve was calculated using the time on the horizontal axis and absorbance value on the vertical axis.

PAMSC wound healing migration assay. PAMSCs at passages 3-5 were digested with 0.25% trypsin, suspended in DMEM supplemented with 10% FBS. Cells were seeded in a 6-well plate at a density of 1×10^5 cells/well. When the cultured cells proliferated to form a full layer, serum-free medium was added for 24 h for cell synchronization. Hydroxyurea (1.8 mmol/l; Sigma-Aldrich; Merck KGaA) was used to inhibit cell proliferation. A scratch was made in the cell monolayer by drawing a sterile P-200 pipette tip across the surface of the culture dish. PBS was used to wash the plate three times. Cell migration into the scratched area was assessed after 0, 12, 24, 48 and 72 h. The areas of cells were measured using Image ProPlus software, version 6.0 (Media Cybernetics, Inc., Rockville, MD, USA). The migration rate of the PAMSCs was calculated as the ratio of the migration area to the original scratch area.

PAMSC Transwell migration assay. PAMSCs at passages 3-5 were cultured in serum-free medium for cell synchronization. PAMSCs were incubated for 12 h with 1.8 mmol/l hydroxyurea and digested with 0.25% trypsin. Following this, cells were suspended in high-glucose DMEM supplemented with 1% FBS and counted ($4\times 10^4/\text{ml}$). Each Transwell-24 plate, containing an upper compartment with 200 μl cell suspension and a lower compartment with 500 μl DMEM supplemented with 10% FBS, was cultured for 4, 8, 12, 24 and 48 h separately. The upper PAMSCs which did not penetrate the membrane

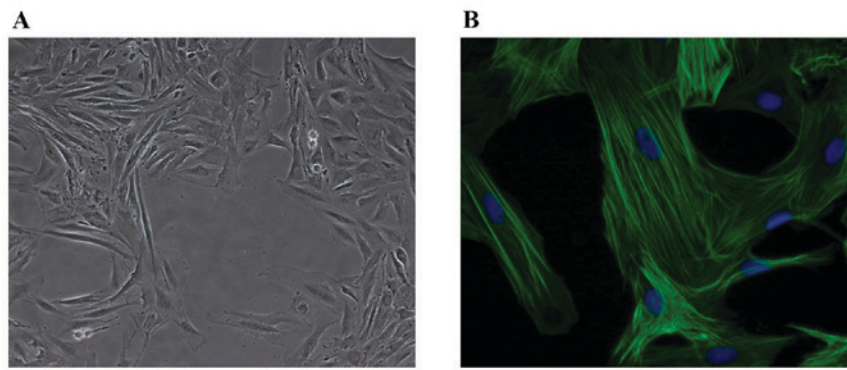


Figure 1. Identification of PSMCs by morphology and immunocytochemistry. (A) Primary PSMCs (magnification, x100). (B) A large amount of SM-actin was observed under a fluorescence microscope. The purity of PSMCs was determined to be high by assessing the association between SM-actin (stained green) and the cell nucleus (stained blue; magnification, x400). PSMCs, pulmonary artery vascular smooth muscle cells; SM-actin, smooth muscle actin.

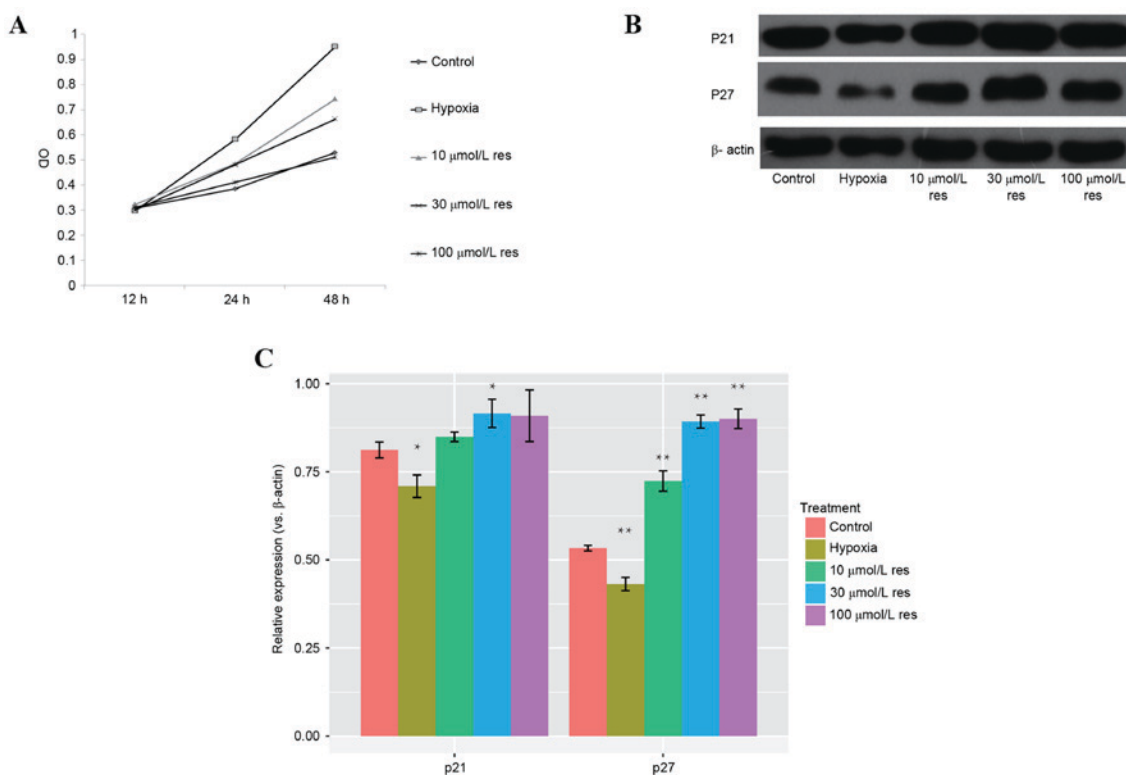


Figure 2. Effects of resveratrol treatment on pulmonary artery vascular smooth muscle cells. (A) The OD value of the treatment groups at 12, 24 and 48 h, as assessed by MTT assay. (B) Representative western blotting images and (C) quantification of p21 and p27 protein expression levels following resveratrol treatment. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. control. Res, resveratrol; OD, optical density.

were removed with a swab. The Transwell semipermeable membrane was washed three times with PBS and fixed with 3.7% paraformaldehyde at room temperature for 5 min, followed by washing three times with PBS again. Nuclear DNA was labelled with 3 $\mu\text{g/ml}$ DAPI, following which cells were washed three times with PBS. Cells were imaged in five random fields under a fluorescence microscope, following which the number of cells penetrating the semipermeable membrane was counted at x100 magnification in triplicate wells of each group.

Protein expression levels of p21, p27, MMP-2, MMP-9, AKT and p-AKT in PSMCs, detected by western blotting. Total

protein was extracted from PSMCs. Briefly, the PSMCs were washed with 1X PBS once and each well in the 12-well cell culture plate was lysed with 100 μl of ice cold radioimmunoprecipitation assay buffer (25 mM Tris-Cl, pH 7.4, 150 mM NaCl, 50 mM KCl, 1% SDS, 2 mM EDTA, 0.5% glycerol, 50 mM NaF) with 1:100 (vol/vol) dilution of the proteinase inhibitor and phosphatase I and II inhibitor mixture (Sigma-Aldrich; Merck KGaA). This was then briefly vortexed and placed on ice for 15 min, spun down at 8,000 \times g for 15 min to pellet the debris and the supernatant was collected for the western blotting. The protein concentration was determined using a Bicinchoninic Acid Protein Assay kit (Sigma-Aldrich; Merck KGaA), according to the manufacturer's instructions.

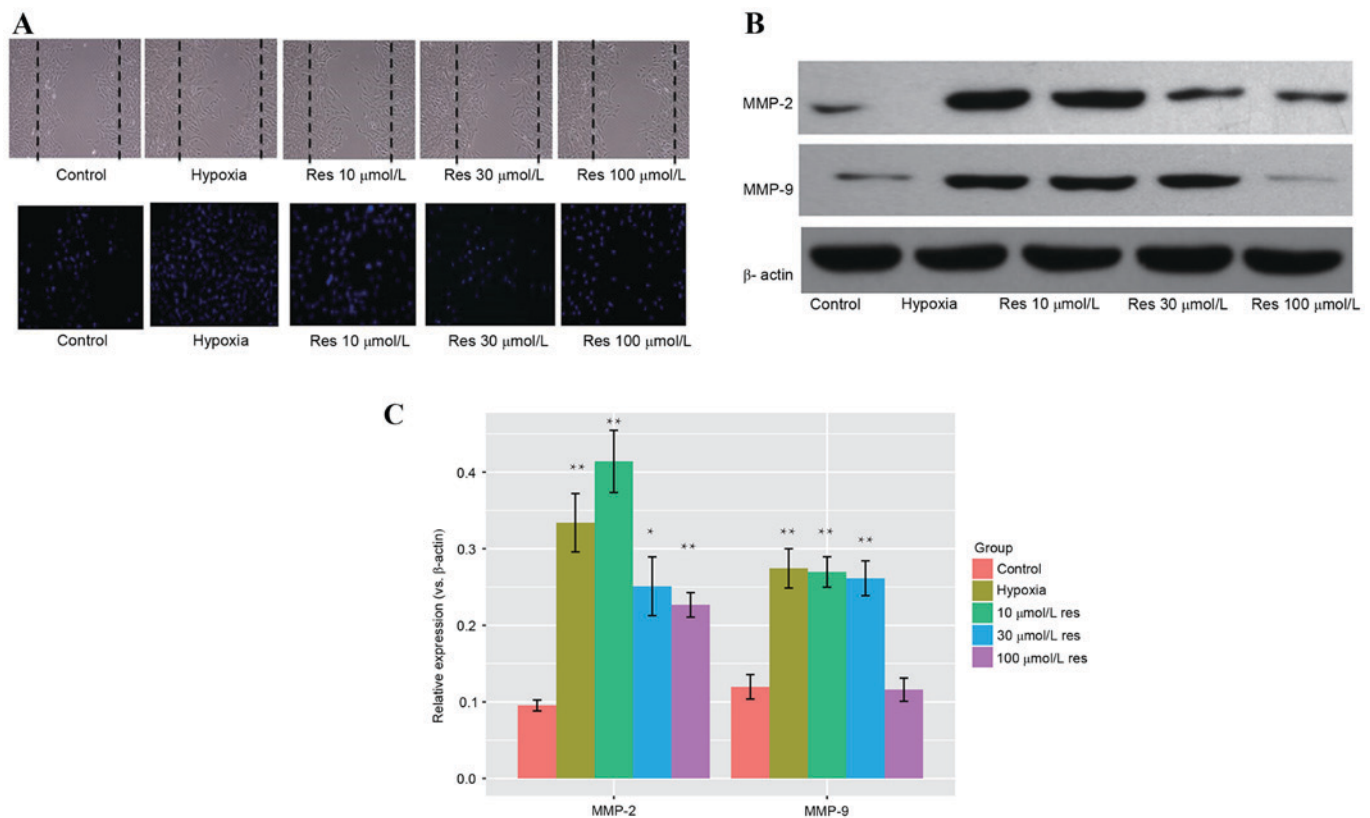


Figure 3. Effects of resveratrol treatment on PAMSC migration, and MMP-2 and MMP-9 protein expression levels. (A) Migration of PAMSCs following each treatment, as assessed by wound healing (magnification, $\times 100$) and Transwell migration (magnification, $\times 200$) assays. (B) Representative western blot images and (C) quantification of protein expression levels of MMP-2 and MMP-9 following resveratrol treatment. Data are presented as the mean \pm standard deviation. * $P < 0.05$ and ** $P < 0.01$ vs. control. Res, resveratrol; PAMSCs, pulmonary artery vascular smooth muscle cells; MMP, matrix metalloproteinase.

Then, 30 μ g protein lysate was boiled with 500 mM DTT in 2X sample buffer for 5 min, separated by 10% SDS-PAGE and transferred onto a nitrocellulose membrane. The membrane was blocked with Tris-buffered saline containing 5% bovine serum albumin (Sigma-Aldrich; Merck KGaA) at room temperature for 30 min, followed by incubation with the appropriate primary antibody (1:1,000) overnight at 4°C. β -actin served as an internal control. Following this, membranes were incubated with a secondary antibody (1:5,000) at room temperature for 1 h. Proteins were visualized by Enhanced Chemiluminescence (EMD Millipore, Billerica, MA, USA).

Statistical analysis. All statistical analysis was performed using SPSS version 12.0 (SPSS, Inc., Chicago, IL, USA). Each assay was performed in triplicate, and data are expressed as the mean \pm standard deviation. Multiple comparisons were evaluated by one-way analysis of variance and Fisher's Least Significant Difference method. $P < 0.05$ was considered to indicate a statistically significant difference.

Results

Culture and identification of primary PAMSCs. PAMSCs were cultured using the tissue explant method. On day 10, cells were observed around the tissue block. Cell fusion occurred on day 15 (Fig. 1A). SM-actin immunofluorescence was identified in PAMSCs, and cell purity was detected to be $>99\%$ by DAPI nuclear staining (Fig. 1B).

Resveratrol treatment inhibits hypoxia-induced proliferation and migration of PAMSCs. The results of the MTT assay indicated that the OD value of the hypoxic group was significantly increased compared with the control group ($P = 0.016$), indicating that hypoxia may promote PAMSC migration. The OD value of resveratrol group markedly decreased in a dose-dependent manner compared with the control group ($P = 0.008$; Fig. 2A). Significantly reduced protein expression levels of p21 and p27 were observed in the hypoxic group by western blot analysis ($P = 0.023$; Fig. 2B). p21 and p27 protein expression levels were increased in the resveratrol group compared with the hypoxic group ($P < 0.05$), and this effect was dose-dependent (Fig. 2C). These results indicated that resveratrol may inhibit hypoxia-induced proliferation of PAMSCs.

Resveratrol treatment inhibits hypoxia-induced migration of PAMSCs. As assessed by Transwell and wound healing migration assays, the migration rate of PAMSCs in the hypoxic group was increased compared with the control group when the intervention factor was considered in each group ($P = 0.012$, Hypoxia vs. Control). Migration of PAMSCs in the resveratrol-treated group was reduced compared with the cells treated with hypoxia ($P < 0.05$), and this effect was dose-dependent (Fig. 3A). Western blot analysis (Fig. 3B) identified significantly increased protein expression levels of MMP-2 and MMP-9 in the hypoxic group when compared with control ($P = 0.002$ for MMP-2; $P = 0.004$ for MMP-9). Protein expression levels of MMP-2 and MMP-9 in the resveratrol group were

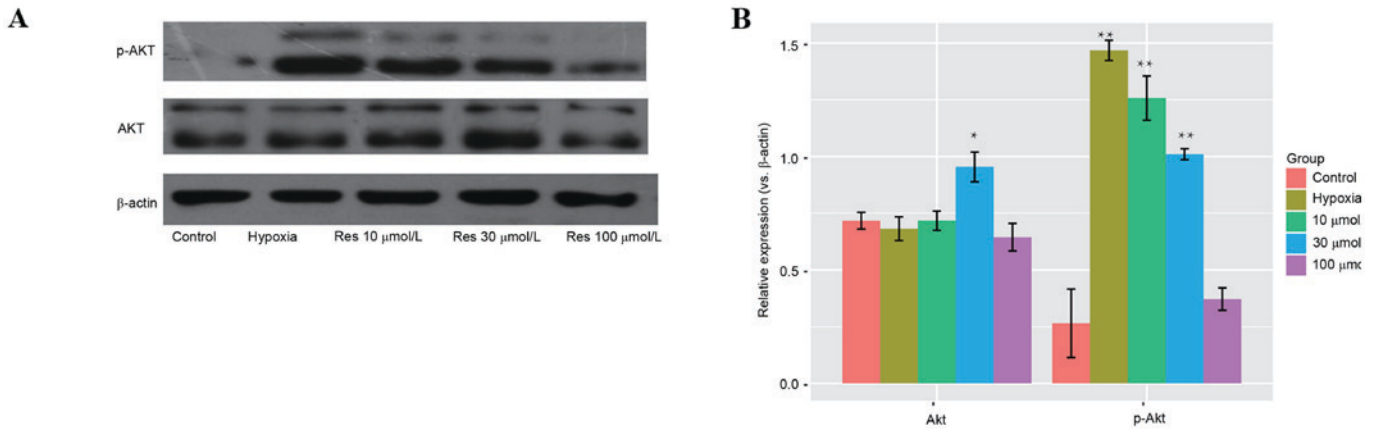


Figure 4. Effects of resveratrol treatment on protein expression levels of AKT and p-AKT in PAMSCs. (A) Representative western blot images and (B) quantification of p-AKT and AKT protein expression levels. Data are presented as the mean \pm standard deviation. * $P<0.05$ and ** $P<0.01$ vs. control. Res, resveratrol; PAMSCs, pulmonary artery vascular smooth muscle cells; AKT, protein kinase B; p-, phosphorylated.

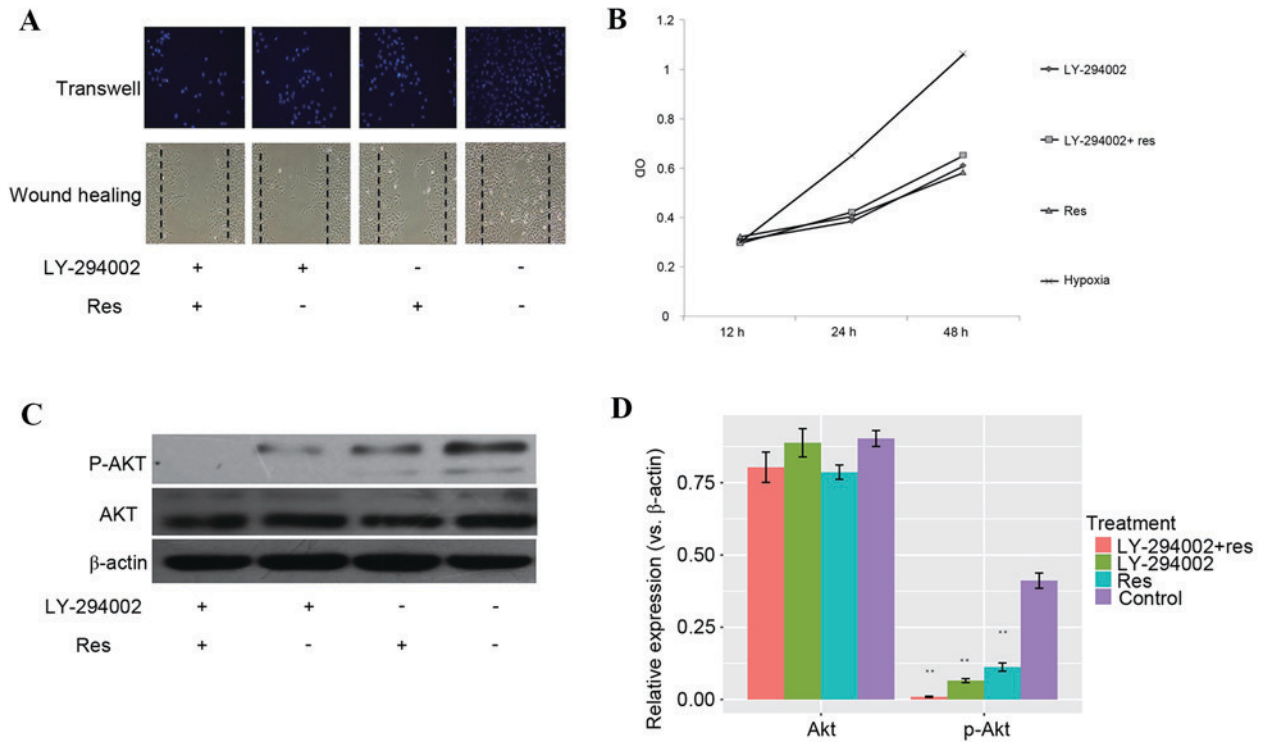


Figure 5. Effects of resveratrol and LY-294002 treatment on PAMSCs. (A) Migration of PAMSCs following each treatment, as assessed by wound healing (magnification, $\times 100$) and Transwell migration (magnification, $\times 200$) assays. (B) Proliferation of PAMSCs 12, 24 and 48 h following treatment with resveratrol in the presence or absence of LY-294002. (C) Representative western blot images and (D) quantification of protein expression levels of AKT and p-AKT treated with resveratrol in the presence or absence of LY-294002. Data are presented as the mean \pm standard deviation. ** $P<0.05$ vs. control. OD, optical density; res, resveratrol; PAMSCs, pulmonary artery vascular smooth muscle cells; AKT, protein kinase B; p-, phosphorylated.

reduced compared with the hypoxic group (MMP-2, $P<0.001$ hypoxia vs. $10 \mu\text{mol/l}$ resveratrol; MMP-9, $P=0.056$ hypoxia vs. $10 \mu\text{mol/l}$ resveratrol), and this effect was dose-dependent (Fig. 3C). These results indicated that resveratrol may inhibit hypoxia-induced migration of PAMSCs.

Resveratrol inhibits activation of the PI3K/AKT signaling pathway. No significant differences in protein expression levels of AKT were observed between the different groups. However, protein expression levels of p-AKT were increased

significantly ($P=0.0021$) in the hypoxic group compared with the 10 and $30 \mu\text{mol/l}$ resveratrol-treated groups, and this effect was dose-dependent (Fig. 4).

Resveratrol inhibits hypoxia-induced proliferation and migration of PAMSCs by inhibiting the PI3K/AKT signaling pathway. LY-294002 is a specific inhibitor of PI3K and significantly inhibits the expression of p-AKT. Following 20 nmol/l LY-294002 treatment, proliferation and migration of PAMSCs were markedly decreased. No significant differences were

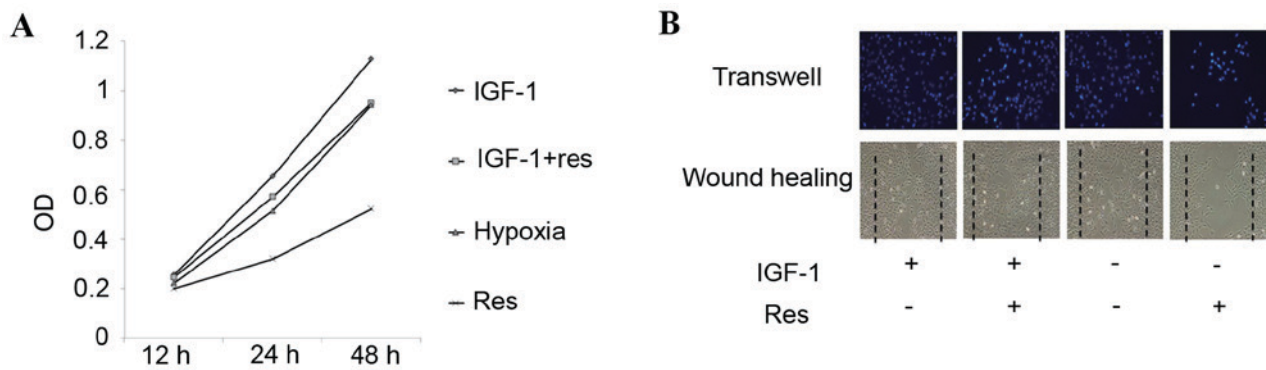


Figure 6. Proliferation and migration of PAMSCs following resveratrol and IGF-1 treatment. (A) Proliferation of PAMSCs 12, 24 and 48 h following treatment with resveratrol and IGF-1 or IGF-1 alone. (B) Migration of the PAMSCs following each treatment, as assessed by wound healing (magnification, x100) and Transwell migration (magnification, x200) assays. PAMSCs, pulmonary artery vascular smooth muscle cells; insulin-like growth factor-1; res, resveratrol.

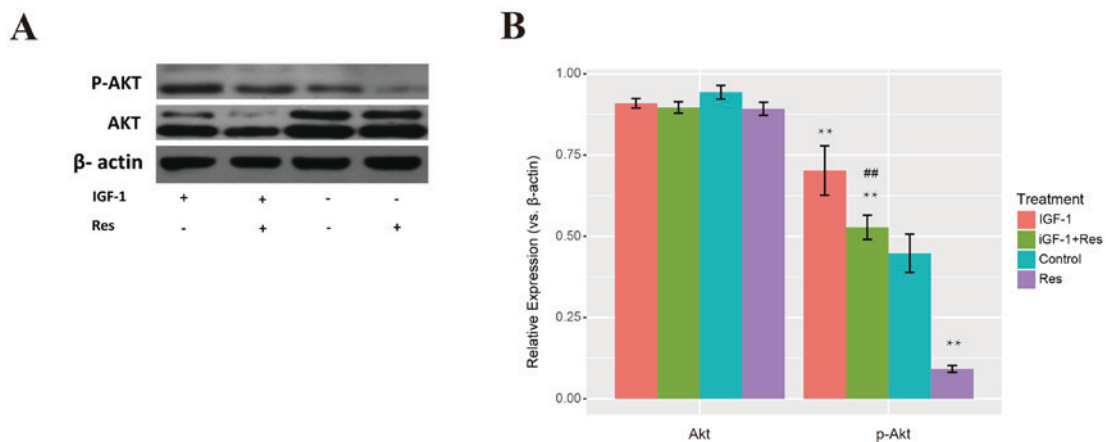


Figure 7. Effects of resveratrol and IGF-1 treatment on AKT and p-AKT protein expression levels in PAMSCs. (A) Representative western blot images and (B) quantification of AKT and p-AKT protein expression levels following IGF-1 treatment or IGF-1 and resveratrol treatment combined. Data are presented as the mean \pm standard deviation. ** $P < 0.01$ vs. control; ## $P < 0.01$ vs. Res treatment. PAMSCs, pulmonary artery vascular smooth muscle cells; AKT, protein kinase B; p, phosphorylated; IGF-1, insulin-like growth factor 1; Res, resveratrol.

observed in migration (Fig. 5A) and proliferation (Fig. 5B) between the resveratrol group treated with LY-294002 and the hypoxic group treated with LY-294002. PAMSCs treated with resveratrol alone seemed to produce the same effect as LY-294002 only treatment on inhibiting hypoxia-induced proliferation, while a combined treatment of resveratrol and LY-294002 did not increase the potency of antiproliferation (Fig. 5B). The western blot of p-Akt displayed a similar pattern (Fig. 5C). Quantification of the protein level of Akt and p-Akt demonstrated that LY-294002 and resveratrol reduced the levels of p-Akt (Fig. 5D), suggesting that resveratrol and LY-294002 may function via a similar mechanism.

IGF-1 is an agonist of PI3K and significantly increases the expression of p-AKT. Following 3 ng/ml IGF-1 treatment, the inhibitory effect on proliferation and migration of PAMSCs was reversed and markedly increased. No significant differences were observed in proliferation and migration between the resveratrol group treated with +IGF-1 and the hypoxic group treated with +IGF-1 in proliferation and migration (Fig. 6A and B). The protein level of p-Akt was significantly suppressed by resveratrol, however, it was markedly increased by IGF-1. The protein level of p-Akt with IGF-1+Res treatment was significantly higher than that of control, while the Res treatment group exhibited

significantly lower levels than the control. The protein level of p-Akt following IGF-1+Res treatment was also significantly increased when compared with Res treatment alone (Fig. 7A and B; $P < 0.01$). Thus, treatment with IGF-1 seemed to counteract the inhibition of resveratrol on p-Akt expression, suggesting that p-Akt may be the pharmacological effector of.

Discussion

PAMSCs are located in pulmonary artery media and serve an important role in vasoconstriction under healthy conditions. The pulmonary arteries of patients with COPD in anoxic conditions are associated with persistent airflow limitation, which induces and activates proliferation and migration of PAMSCs (17). Consequently, proliferating and migrating PAMSCs secrete inflammatory factors, causing cascade amplification of the inflammatory reaction and increasing levels of components of the extracellular matrix simultaneously. These factors lead to remodeling of the pulmonary arteries and luminal stenosis, resulting in development of pulmonary hypertension (18,19).

Resveratrol is a type of polyphenol with pleiotropic biology that is extracted from red wine, and has gained increasing

attention due to its protective properties for cardiac-cerebral vessels (20). Follow-up studies have identified that resveratrol has cardioprotective effects, and may inhibit tumor cell growth and attenuate diabetes-associated complications by its anti-inflammatory and antioxidant properties (21-23). Csiszar *et al* (3) first identified that resveratrol may inhibit the development of monocrotaline-induced pulmonary hypertension; however, the underlying mechanisms are not clear (3). Previous studies demonstrated that resveratrol may improve the function of rat pulmonary artery endothelium by increasing the expression of NO to decrease the effect of oxidative stress, inhibit inflammatory reactions and inhibit cell proliferation and vascular remodeling (3,24). Resveratrol may reverse the dysfunction of rat pulmonary artery vessels and inhibit cardiomyocyte hypertrophy (25,26). The present study demonstrated that resveratrol may inhibit proliferation and migration of PASMCs, which may be the mechanism underlying the inhibitory effect on the development of pulmonary hypertension. In addition, resveratrol inhibited cell proliferation of PASMCs induced by hypoxia in a dose-dependent manner. Protein expression levels of p21 and p27, which are established cyclin-dependent kinase inhibitors (27,28), were rescued from hypoxia following treatment with resveratrol, suggesting the potential role of resveratrol in regulating cell cycles. MMPs serve important roles in proliferation and metastasis of smooth muscle cells, and provide space for PASMCs to migrate and proliferate around surrounding tissues. On the other hand, MMPs may increase cell migration and proliferation by activating the PI3K/AKT signaling pathway (29,30). The results of the present study demonstrated that resveratrol significantly inhibits protein expression levels of MMP-2 and MMP-9, which may be one of the underlying mechanisms inhibiting proliferation and migration of PASMCs.

The PI3K/AKT signaling pathway serves a key role in proliferation and migration of PASMCs and phenotype switch (31). p-AKT may further activate the mTOR/P70S6K signaling pathway, increase the expression of p-P70S6K and increase proliferation and migration of PASMCs (32). Chen *et al* (33) demonstrated that angiotensin II may increase the expression of p-AKT in rat pulmonary artery tissues, leading to the formation of pulmonary hypertension. Goncharova *et al* (34) further demonstrated that activation of the PI3K/AKT signaling pathway in PASMCs via formation of the mTOR complex led to increased transcription and translation of genes associated with cell proliferation, eventually leading to proliferation of PASMCs. These results were consistent with those of the present study, where resveratrol was demonstrated reduce p-AKT and AKT protein expression levels, thereby inhibiting proliferation and migration of PASMCs. In addition, previous studies have demonstrated that resveratrol has synergistic functions with the AKT phosphorylation inhibitor LY-294002; however, IGF-1 may counterbalance the activation of AKT (24-26). This was observed in the present study as protein expression levels of p-Akt were downregulated despite considerable basal expression of AKT in each treatment group, indicating that resveratrol may reduce the expression of AKT and abrogate its phosphorylation.

In conclusion, the present study was based on previous studies which have demonstrated that resveratrol may inhibit proliferation and migration of PASMCs. It was demonstrated

that resveratrol may inhibit proliferation and migration of PASMCs by blocking the PI3K/AKT signaling pathway. The present study provided a novel perspective of the underlying mechanisms of resveratrol treatment on resistance to HPH.

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