Introduction

Individuals living at high altitude either permanently or temporarily may develop pulmonary arterial hypertension (PAH), which in turn may lead to right ventricular hypertrophy and eventually to heart failure (1, 2). The imbalance between apoptosis and proliferation of pulmonary artery smooth muscle cells (PASMCs) resulting from progressive vascular remodeling is a major factor contributing to the complicated pathogenesis of PAH (3–5). It is known that hypoxia will upset the balance between apoptosis and proliferation of PASMCs, leading to thickening of pulmonary artery walls and vascular remodeling and thus to various high-altitude diseases, such as high altitude pulmonary edema and heart diseases (6–9), that are associated with significant morbidity and mortality in individuals living at high altitude. For this reason, a great deal of research has focused on drugs effective in suppressing proliferation and mediating apoptosis of PASMCs (10, 11).

Antiproliferative Effect of Echinacoside on Rat Pulmonary Artery Smooth Muscle Cells Under Hypoxia

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Abstract. The main purpose of this study is to evaluate the effect of echinacoside (ECH) on hypoxia-induced proliferation of rat pulmonary artery smooth muscle cells (PASMCs) and the underlying mechanism. PASMCs were incubated under normoxia (nor), hypoxia (hyp), hypoxia + 0.35 mM ECH (hyp + ECH₀.₃₅), or hypoxia + 0.4 mM ECH (hyp + ECH₀.₄) for 24 h. Cell viability was assessed by MTS assays. The morphology of apoptosis was observed by DAPI staining, and apoptosis was quantified by flow cytometric analysis. Caspase-3 activity was determined by immunohistochemistry and real-time PCR, and the expressions of HIF-1α, Bax, Bcl-2, and Fas were determined by real-time PCR. Hypoxia induced significant proliferation of PASMCs, which could be inhibited by ECH in a concentration-dependent manner. This was associated with apoptosis of PASMCs. Z-DEVD-FMK could partly reduce the suppression effect of ECH; protein and gene expression of caspase-3 were significantly higher in the hyp + ECH₀.₄ and hyp + ECH₀.₃₅ groups. ECH significantly increased the expressions of Bax and Fas, but decreased the expressions of Bcl-2 and HIF-1α. ECH could inhibit hypoxia-induced proliferation of rat PASMCs, which is associated with apoptosis of PASMCs and improvement of hypoxia. ECH might be a potential agent for prevention and treatment of hypoxia-induced PAH.

Keywords: Tibetan herb, pulmonary arterial hypertension, high altitude, proliferation of rat pulmonary artery smooth muscle cell (PASMC), apoptosis

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Echinacoside (ECH) (Fig. 1) is a phenylethanoid glycoside found in various species, such as the Tibetan herb *Lagotis brevituba* Maxim and the traditional Chinese medicine *Cistanche tubulosa* (12, 13). *Lagotis brevituba* is a species of *Lagotis* Gaertn. spp belonging to Scrophulariaceae and grows widely at an altitude over 3000 meter in the Qinghai-Tibet Plateau of China. It can be used to treat various diseases, including visceral ‘heat’, heat-toxicity in the blood system, tidal fever, hypertension, acute or chronic hepatitis, systemic fever, nephritis, pneumopathy, atherosclerosis, irregular menstruation, toxicosis, and heart heat syndrome (14). *Cistanche tubulosa* is a species of *Cistanche* genus belonging to Orobanchaceae family and grows in the northwest of China, North Africa, Arabia, as well as other Asian countries (15). It has been used as a traditional Chinese medicine with anti-aging properties in the treatment of senile dementias, such as vascular dementia and Alzheimer’s type dementia (14). As a naturally derived compound with multiple hydroxyl phenolic structure, ECH has been reported to have various pharmacological activities, such as antioxidative, antiinflammatory, neuroprotective, hepatoprotective, and nitric oxide radical–scavenging activities (16). It has also been reported to elicit endothelium-dependent relaxation in rat thoracic aorta and cure cardiovascular diseases (17). Our previous study found that ECH could induce vasorelaxation of rat pulmonary artery with either intact or denuded endothelium. However, to the best of our knowledge, there is no published study addressing its effects on the proliferation of PASMCs.

The aims of the present study are to investigate: 1) whether ECH can inhibit hypoxia-induced proliferation of rat PASMCs; 2) whether the inhibitory effect, if any, is associated with apoptosis of PASMCs; and 3) the underlying mechanism responsible for the effect of ECH under hypoxia.

**Materials and Methods**

**Drugs and reagents**

ECH (20121104) was kindly provided by Dr. Pengfei Tu (Peking University, Beijing, China), and deposited at the Department of Natural Medicine, School of Pharmaceutical Sciences, Peking University Health Science Center. The purity of the ECH was assessed by an HPLC analysis (15). Dimethyl sulfoxide (DMSO) was purchased from Solarbio Science & Technology Co., Ltd. (Beijing, China); high glucose Dulbecco’s modified Eagle’s medium (DMEM), fetal bovine serum (FBS), and trypsin from Gibco BRL Co., Ltd. (Gaithersburg, MD, USA); inhibitor of caspase-3 Z-Asp(O-Me)-Glu(O-Me)-Val-Asp(O-Me) fluoromethyl ketone (Z-DEVD-FMK) from Sigma Chemical Co. (St. Louis, MO, USA); and all inorganic salts from Beijing Chemical Reagent Co., Ltd. (Beijing, China). ECH was dissolved in DMSO, and preliminary experiments showed that DMSO less than 0.1% (v/v) had no effect on cells. The 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium (MTS) assay was performed using a CellTiter 96® AQueous One Solution Cell Proliferation Assay kit from Promega Co., Ltd. (Madison, WI, USA). Annexin V-FITC/PI Apoptosis Detection Kit was purchased from Molecular Biology Resources, Inc. (Milwaukee, WI, USA); antibody against cleaved caspase-3 and monoclonal mouse anti-rat primary SMA antibody from Boshide Biotechnology Co., Ltd. (Wuhan, China); Trizol reagent, high capacity cDNA Reverse Transcription Kit and SYBR® Select Master Mix Kit from Invitrogen Corp. (Carlsbad, CA, USA); and qPCR primers from Qiagen company (Germany), including Fas (Cat. No. QT00196595), Bcl-2 (Cat. No. QT00184863), Bax (Cat. No. QT00184863), Bcl-2 (Cat. No. QT00184863), Bax (Cat. No. QT01081752), HIF-1α (Cat. No. QT00182532), 18S (Cat. No. QT00199374), and caspase-3 (Cat. No. QT01794429).

**Experiment animals**

All procedures and protocols were approved by the Animal Care and Use Committee of the Medical College of Qinghai University. Male Wistar rats, 6 – 8-week-old, were purchased from the Animal Center of Lanzhou University (Gansu, China) and maintained on a standard laboratory diet and tap water ad libitum at an ambient temperature of 22°C ± 2°C and a relative humidity of 45% – 55% throughout the experiments.

**Isolation and culture of PASMC**

Distal pulmonary arteries were isolated from male Wistar rats, and the endothelium and adventitia were removed carefully. Then the pulmonary arterial smooth muscles were minced into 2-mm pieces and added to the flasks immediately, maintained at 37°C in a humidified atmosphere of 5% CO₂–95% air for about 3 – 4 hours, and then cultured with high-glucose DMEM supplemented with 20% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin for another 5 – 7 days. When cells came out, one-half of the culture media was replaced with...
fresh media. When cells reached 80% confluence on the 9th or 10th day, they were harvested with 0.125% trypsin and seeded into flasks (1:2 ratio) containing high-glucose DMEM supplemented with 10% FBS, 100 U/mL penicillin, and 100 U/mL streptomycin. When the confluent PASMCS manifested typical “hill and valley” features, they were positive for immunochemical staining. Cells were subcultured after detachment with 0.125% trypsin twice a week, and the culture medium was replaced every 48 h. Cells from 3 to 8 passages were used for all experiments.

When cells reached 80% confluence, the culture media were replaced with serum-free DMEM. After an incubation of 24 h, cells were exposed to one of four treatment conditions: 1) normoxia group (nor group), in which cells were incubated with low-serum (2% FBS) DMEM under normoxia (5% CO₂–95% air) for 24 h; 2) hypoxia group (hyp group), in which cells were incubated with low-serum (2% FBS) DMEM in an air-tight hypoxic chamber (Thermo Electron, Waltham, CA, USA) containing 3% O₂–5% CO₂–92% N₂ for 24 h (18); 3) hypoxia + ECH (0.35 mM) group (hyp + ECH₀.₃₅ group), in which cells were incubated with low-serum (2% FBS) DMEM supplemented with 0.35 mM ECH for 24 h under hypoxia; and 4) hypoxia + ECH (0.4 mM) group (hyp + ECH₀.₄ group), in which cells were incubated with low-serum (2% FBS) DMEM supplemented with 0.4 mM ECH for 24 h under hypoxia.

Identification of PASMCS by immunohistochemistry

Cells cultured on glass coverslips were fixed in 4% paraformaldehyde and blocked with 3% hydrogen peroxide for 15 min and then incubated with mouse anti-rat primary SMA antibody at a dilution of 1:200 overnight at 4°C. After washing three times in PBS for 10 min, cells were incubated with goat anti-mouse secondary antibody at room temperature for 45 min, washed again three times in PBS for 10 min, and visualized with diaminobenzidine (DAB).

MTS

Cell viability was assessed by MTS assays, cells were seeded in 96-well microplates at a density of 1 × 10⁴ cells/well in 100 μl of culture medium, and subsequently 20 μl of CellTiter 96® AQueous One Solution was added. Then cells were incubated for 1 – 3 h, and the absorbance was quantified by spectrophotometry at 490 nm using a Microplate reader (ELX800; Bio-Tek, VT, USA). Survival rate was calculated according to the following formula: survival rate (%) = optical density (OD) value of experimental group / OD value of control (nor) group × 100%.

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Determination of the effect of caspase-3

The role of the inhibitor of caspase-3: The cells were divided into seven groups: 1) normoxia group (nor group), 2) hypoxia group (hyp group), 3) hypoxia + ECH (0.35 mM) group (hyp + ECH₀.₃₅ group), 4) hypoxia + ECH (0.4 mM) group (hyp + ECH₀.₄ group), 5) hypoxia + Z-DEVD-FMK (20 μM) group (hyp + Z-DEVD-FMK group), (6) hypoxia + ECH (0.35 mM) + Z-DEVD-FMK (20 μM) group (hyp + ECH₀.₃₅ + Z-DEVD-FMK group); and (7) hypoxia + ECH (0.4 mM) + Z-DEVD-FMK (20 μM) group (hyp + ECH₀.₄ + Z-DEVD-FMK group). After the treatment, cell viability was assessed by MTS assay.

Measurement of caspase-3 activity by immunohistochemistry: Cells cultured on glass coverslips were fixed in 4% paraformaldehyde and blocked with 3% hydrogen peroxide for 15 min and then incubated with rabbit anti-rat caspase-3 antibody at a dilution of 1:200 overnight at 4°C. After washing three times in PBS for 10 min, cells were incubated with goat anti-rabbit secondary antibody at room temperature for 45 min, washed again three times in PBS for 10 min, and visualized with DAB. Caspase-3 activity was quantified by measuring the mean OD using Image Pro-Plus 6.0 software.
Statistics
Data were expressed as the mean ± S.D. When appropriate, statistical differences were assessed by Dunnett’s test or Student-Newman-Keuls for multiple comparisons after one-way analysis of variance (ANOVA). A probability level of \( P < 0.05 \) was considered as statistically significant.

Results

Identification of rat PASMCs
Spindle cells were densely packed and stained positive for PASMCs at low magnification. The purity of PASMCs was > 95% (Fig. 2A), and brown-stained myofilaments were clearly visible in the cytoplasm at high magnification (Fig. 2B).

Fig. 2. Rat PASMCs were positive for immunochemical staining with specific SMA antibody. A) Spindle cells were densely packed and stained positive for PASMCs at low magnification. B) Brown-stained myofilaments were clearly visible in the cytoplasm at high magnification.

Fig. 3. ECH inhibited hypoxia-induced proliferation of rat PASMCs. A) Cells in normoxia. Elongated spindle shaped cells were clearly observed in a sparse single layer. B) Cells in hypoxia (3% O\(_2\)–5% CO\(_2\)–92% N\(_2\)). Hypoxia-induced proliferation led to the formation of several layers of cells, and the shape of cells was not clear. C and D) Cells treated by ECH (0.35 or 0.4 mM). E) Hypoxia induced significant proliferation of rat PASMCs (n = 6). ECH could reduce the survival rate of rat PASMCs in a concentration-dependent manner (n = 6). *\( P < 0.01 \) vs. the normoxia (nor) group, †\( P < 0.01 \) vs. the hyp group, ‡\( P < 0.01 \) vs. the hyp + ECH\(_{0.35}\) group.

Fig. 4. DAPI staining of rat PASMCs. A) Cells in hypoxia (control). Most cells were normal and DAPI-negative and round-shaped, and their nuclei were stained with a less bright blue fluorescence. B and C) cells in hyp + ECH\(_{0.35}\) group and hyp + ECH\(_{0.4}\) group. The cells displayed fluorescence with higher intensity than normal cells due to the highly condensed chromatin. Nuclear shrinkage, chromatin condensation and apoptotic bodies, the hallmarks of apoptosis, were observed.
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MTS
PASMCs were incubated with ECH (0, 0.35, or 0.4 mM) under normoxia or hypoxia for 24 h, and cell viability was assessed by MTS assay. Fig. 3, A, B, and E showed that hypoxia induced significant proliferation of PASMCs. However, such proliferation of PASMCs could be inhibited by ECH in a concentration dependent manner (Fig. 3: C, D, and E), as it was noted that cell viability was significantly lower in PASMCs cultured with 0.4 mM ECH than in PASMCs cultured with 0.35 mM ECH.

DAPI staining for morphological detection of apoptosis
Apoptosis is initially characterized by morphological changes, such as chromatin condensation and nuclear fragmentation which implies DNA damage (19). DAPI is a fluorescent stain used to visualize nuclear DNA for morphological changes of apoptosis (20). The results indicated that most cells in the control group (hyp group) were normal and DAPI-negative. Normal rat PASMCs were round-shaped, and their nuclei were stained with a less bright blue fluorescence (Fig. 4A). However, cells treated with ECH (0.35 or 0.4 mM) displayed fluorescence with higher intensity than normal cells due to the highly condensed chromatin. Nuclear shrinkage, chromatin condensation, and apoptotic bodies, the hallmarks of apoptosis, were observed in the cells (Fig. 4: B and C). Thus, most cells died primarily through apoptosis by the treatment of ECH.

Annexin V-FITC apoptosis assay
Flow cytometric analysis (Fig. 5: A and B) revealed a concentration-dependent increase in apoptotic cells after treatment with ECH. The treatment of 0.35 mM ECH resulted in an increase of both early (9.30%) and late (4.03%) apoptotic cells as compared with the hyp group, which however could be further increased with the treatment of higher dose (0.4 mM) of ECH (36.90% and 7.76%, respectively). Thus, the apoptosis-inducing effect of PASMCs plays a key role in the suppression effect of PASMCs under hypoxia. Again, the apoptosis was significantly higher in PASMCs cultured with 0.4 mM ECH than in PASMCs cultured with 0.35 mM ECH.

Caspase-3 activity
Figure 6 shows that hypoxia induced significant proliferation of rat PASMCs, which however could be inhibited by ECH in a concentration-dependent manner. However, the suppression effect of ECH could be partly abrogated by the caspase-3–specific inhibitor Z-DEVD-FMK. It seemed that the caspase-3 signal pathway played a key role in the suppression of ECH on rat PASMCs under hypoxia.

The protein and gene expressions of caspase-3 were significantly higher in the hyp + ECH0.35 group than in the hyp + ECH0.4 group, both of which were significantly higher than the hyp or nor groups (Figs. 7 and 8A). These results indicated that ECH could increase the expression of active caspase-3 in a concentration-dependent manner, thus leading to apoptosis of rat PASMCs under hypoxia.

Expressions of Bax, Bcl-2, Fas, and HIF-1α
Real-time PCR results showed that the expressions of Bax and Fas were lower (Fig. 8: B and D), but the expressions of Bcl-2 and HIF-1α were higher (Fig. 8: C and E) in the hyp group than in the nor group. It was also noted that ECH significantly increased the expression of Bax and Fas (Fig. 8: B and D), but decreased the expression of Bcl-2 and HIF-1α (Fig. 8: C and E). Thus, ECH inhibited hypoxia-induced proliferation of rat PASMCs partly by reducing the expression of HIF-1α and Bcl-2, and increasing the expression of Bax and Fas.

Discussion
To the best of our knowledge, this is the first study evaluating the effect of ECH on hypoxia-induced proliferation of rat PASMCs. We found that ECH had an inhibitory effect on hypoxia-induced proliferation of rat PASMCs, which was associated with the induction of apoptosis of PASMCs. This was achieved by increasing the expression of caspase-3 or activating caspase-3 to regulate apoptosis-inducing and -inhibiting factors such as Fas, Bax, and Bcl-2. It showed that ECH decreased the expression of HIF-1α, a specific factor responsible for hypoxic responses (21), indicating that ECH could improve hypoxia.

We found that rat PASMCs exposed to hypoxia for 24 h exhibited significant proliferation, which was consistent with previous studies (22, 23). However, such proliferation of rat PASMCs induced by hypoxia was inhibited by ECH in a concentration-dependent manner. It showed that the expression of HIF-1α was significantly increased in PASMCs exposed to hypoxia for 24 h, but decreased by ECH (0.35 and 0.4 mM). These results indicated that ECH mediated the downregulation of HIF-1α in rat PASMCs under hypoxia. HIF-1α has been described as a key regulator in the adaptation to hypoxia, and mediates the proliferation of PASMCs in response to hypoxia (21, 24). Partial HIF-1α deficiency significantly alleviated pulmonary vasoconstriction, vascular remodeling, and hypertension (25). In this study, we found that ECH inhibited hypoxia-induced proliferation of PASMCs partly by decreasing the expression of HIF-1α.

Pharmacological interventions in PAH mainly focus on the modulation of smooth muscle cell proliferation...
and migration, activity of matrix metalloproteinases, and cell apoptosis, which is a major form of death (1). Apoptosis is initially characterized by morphological changes such as chromatin condensation and nuclear fragmentation that imply DNA damage. In this study, nuclear shrinkage, chromatin condensation and apoptotic bodies, the hallmarks of apoptosis, were observed in cells treated with 0.35 or 0.4 mM ECH. Additionally,
the flow cytometric analysis showed that ECH (0.35 and 0.4 mM) could increase the number of early apoptotic PASMCs and late apoptotic PASMCs under hypoxic conditions. These observations indicate that induction of apoptosis of rat PASMCs plays a key role in the inhibitory effect of ECH on rat PASMCs under hypoxia.

Caspase-3 is one of the critical executioners in the regulation pathway of PASMC proliferation and apoptosis (26, 27). Procaspase-3 could be directly or indirectly activated by other factors, and the active enzyme is composed of two subunits of 17 kDa and 12 kDa (28, 29). We investigated the effect of caspase-3 in the suppression effect of ECH using the caspase-3–specific inhibitor Z-DEVD-FMK. The result indicated that the suppressive effect of ECH could be partly abrogated by Z-DEVD-FMK; thus the caspase-3 signal pathway played a key role in the suppression of ECH on rat PASMCs under hypoxia. However, more research is needed to detect the mRNA and protein level of caspase-3. We found that ECH increased the expression of active caspase-3, indicating that ECH could influence the expression or activation of caspase-3 to induce apoptosis of rat PASMCs under hypoxia.

Bcl-2 family proteins play a key role in cell apoptosis (30). Bcl-2 and Bax are representatives of Bcl-2 family proteins and have similar structures but antagonistic functions. Previous research showed that Bax and Bcl-2 could regulate mitochondrial outer membrane permeability to mediate activation of caspase-3 (31 – 33). When Bax is activated, the N terminal is exposed and it translocates to the mitochondrial outer membrane, changing the permeability of the mitochondrial outer

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Fig. 8. Real-time PCR results of caspase-3, Bax, Bcl-2, Fas, and HIF-1α. The gene expression of A) caspase-3, B) Bax, C) Bcl-2, D) Fas, and E) HIF-1α. The expressions of Bax and Fas were lower, but the expressions of Bcl-2 and HIF-1α were higher in the hyp group than in the nor group. ECH (0.35 and 0.4 mM) significantly increased the expressions of caspase-3, Bax, and Fas, but decreased the expressions of Bcl-2 and HIF-1α to a different level (n = 3, *P < 0.05 vs. the nor group; #P < 0.05 vs. the hyp group; #P < 0.05 vs. the hyp + ECH16 group).
membrane, leading to the loss of \( A_\text{ym} \) and cyt-C release (34). On the contrary, Bcl-2 exerts the anti-apoptotic action at or before the processing of certain caspases to their catalytically active forms (35). The results of our study showed that rat PASMCs exposed to hypoxia for 24 h exhibited a significant reduction in Bax expression and an overexpression of Bcl-2. ECH could reduce the overexpression of Bcl-2 and increases the expression of Bax. Thus, ECH at least in part mediated the expression of Bax and Bcl-2 to induce apoptosis of PASMCs under hypoxia.

Fas is a kind of uni-transmembrane glycosylated receptor protein on the cell surface and belongs to the family of tumor necrosis factor and nerve growth factor receptors that can induce cell apoptosis (36). This study showed that apoptosis of rat PASMCs was promoted with the stimulation of Fas expression in the hyp + ECH\(_{0.35}\) and hyp + ECH\(_{0.4}\) groups. This might be one of the mechanisms by which ECH modulates the apoptosis of rat PASMCs and attenuates hypoxic pulmonary vascular structural remodeling.

In conclusion, ECH inhibited hypoxia-induced proliferation of rat PACMCs in a concentration-dependent manner, and the antiproliferative effect of ECH was associated with the induction of apoptosis of PASMCs via the caspase-dependent mitochondrial and death receptor pathways and the improvement of hypoxia.

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