

Original Article

# Echinacoside induces rat pulmonary artery vasorelaxation by opening the NO-cGMP-PKG-BK<sub>Ca</sub> channels and reducing intracellular Ca<sup>2+</sup> levels

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**Aim:** Sustained pulmonary vasoconstriction as experienced at high altitude can lead to pulmonary hypertension (PH). The main purpose of this study is to investigate the vasorelaxant effect of echinacoside (ECH), a phenylethanoid glycoside from the Tibetan herb *Lagotis breviflora* Maxim and *Cistanche tubulosa*, on the pulmonary artery and its potential mechanism.

**Methods:** Pulmonary arterial rings obtained from male Wistar rats were suspended in organ chambers filled with Krebs-Henseleit solution, and isometric tension was measured using a force transducer. Intracellular Ca<sup>2+</sup> levels were measured in cultured rat pulmonary arterial smooth muscle cells (PASMCs) using Fluo 4-AM.

**Results:** ECH (30–300 μmol/L) relaxed rat pulmonary arteries precontracted by noradrenaline (NE) in a concentration-dependent manner, and this effect could be observed in both intact endothelium and endothelium-denuded rings, but with a significantly lower maximum response and a higher EC<sub>50</sub> in endothelium-denuded rings. This effect was significantly blocked by L-NAME, TEA, and BaCl<sub>2</sub>. However, IMT, 4-AP, and Glibenclamide did not inhibit ECH-induced relaxation. Under extracellular Ca<sup>2+</sup>-free conditions, the maximum contraction was reduced to 24.54%±2.97% and 10.60%±2.07% in rings treated with 100 and 300 μmol/L of ECH, respectively. Under extracellular calcium influx conditions, the maximum contraction was reduced to 112.42%±7.30%, 100.29%±8.66%, and 74.74%±4.95% in rings treated with 30, 100, and 300 μmol/L of ECH, respectively. After cells were loaded with Fluo 4-AM, the mean fluorescence intensity was lower in cells treated with ECH (100 μmol/L) than with NE.

**Conclusion:** ECH suppresses NE-induced contraction of rat pulmonary artery via reducing intracellular Ca<sup>2+</sup> levels, and induces its relaxation through the NO-cGMP pathway and opening of K<sup>+</sup> channels (BK<sub>Ca</sub> and K<sub>IR</sub>).

**Keywords:** Tibetan herb; echinacoside; pulmonary hypertension; high altitude; vasorelaxation; NO; BK<sub>Ca</sub>; K<sub>IR</sub>; artery ring; pulmonary arterial smooth muscle cells

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## Introduction

Sustained pulmonary vasoconstriction as experienced at high altitude can lead to pulmonary hypertension (PH) and medial hypertrophy<sup>[1]</sup>, which have been identified as the main cause of right ventricular hypertrophy and heart failure<sup>[2]</sup>. Individuals living at high altitude, either temporarily or permanently, may present mild to moderate pulmonary hypertension, and those with greater susceptibility to hypoxia may have exaggerated pulmonary vasoconstriction, leading to various high-

altitude diseases that are associated with significant morbidity and mortality, such as high-altitude pulmonary edema and heart diseases<sup>[3]</sup>. Echinacoside (ECH) (Figure 1) is a phenylethanoid glycoside found in a variety of Chinese herbs such as the Tibetan herb *Lagotis breviflora* Maxim and *Cistanche tubulosa*<sup>[4,5]</sup>. *Lagotis breviflora* Maxim is a species of *Lagotis* spp belonging to the Scrophulariaceae and grows widely at an altitude over 3000 meter in the Qinghai-Tibet Plateau. ECH has various desirable pharmacological characteristics, such as antioxidative, anti-inflammatory, neuroprotective, hepatoprotective, and nitric oxide (NO) radical-scavenging properties<sup>[6]</sup>. It can also elicit endothelium-dependent relaxation in rat thoracic aortic rings and cure cardiovascular diseases<sup>[7]</sup>. How-

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ever, to the best of our knowledge, there has not been a study investigating its effects on the vascular tone in pulmonary arteries.

The purposes of this study are as follows: (1) to explore whether ECH induces *in vitro* vasorelaxation in rat pulmonary arteries precontracted with noradrenaline (NE), and whether this effect, if any, is endothelium-dependent or acts directly upon vascular smooth muscle; (2) to investigate the effect of ECH on the extracellular calcium influx and intracellular calcium release in rat pulmonary arterial smooth muscle cells (PASMCs); and (3) to identify possible pathways and K<sup>+</sup> channels involved in ECH-induced relaxation.

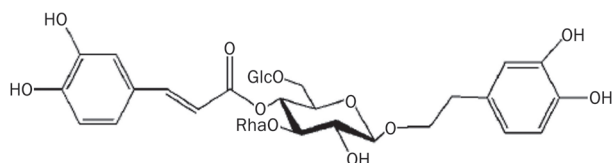


Figure 1. The chemical structure of echinacoside.

## Materials and methods

### Reagents

ECH was kindly provided by Dr Peng-fei TU of Peking University (Beijing, China), with a purity of more than 98% as determined by high-performance liquid chromatography. Dimethyl sulfoxide (DMSO) was purchased from Solarbio Science & Technology Co, Ltd (Beijing, China); NE, acetylcholine (ACh, ≥99%), indomethacin (IMT), N<sup>ω</sup>-Nitro-L-arginine methyl ester hydrochloride (L-NAME), tetraethylammonium chloride (TEA), barium chloride (BaCl<sub>2</sub>), 4-aminopyridine (4-AP), and glibenclamide (Gli) from Sigma Chemical Co (St Louis, MO, USA); high-glucose Dulbecco's modified Eagle's medium (DMEM), fetal bovine serum (FBS), and trypsin from Gibco BRL Co, Ltd (Gaithersburg, MD, USA); mouse anti-rat primary α smooth muscle actin (α-SMA) antibody and goat anti-mouse secondary antibody from Boshide Biotech Co, Ltd (Wuhan, China); Fluo Calcium Indicators (Fluo 4-AM) from Invitrogen Corp (Carlsbad, CA, USA); and all inorganic salts from Beijing Chemical Reagent Co, Ltd (Beijing, China). IMT was dissolved in DMSO and 5% NaH<sub>2</sub>PO<sub>4</sub>, Gli, Fluo 4-AM, and ECH were dissolved in DMSO, and all other reagents were dissolved in Krebs-Henseleit (KH) or PBS solution. Preliminary experiments showed that DMSO at less than 0.1% (*v/v*) had no effect on the tension development of isolated pulmonary arterial rings.

### Experimental 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 weeks old, 250–300 g body weight, were purchased from the Animal Center of Lanzhou University (Lanzhou, China) and maintained on a standard laboratory diet and tap water *ad libitum* at an ambient temper-

ature of 22±2°C and a relative humidity of 45%–55% throughout the experiments.

### *In vitro* pulmonary artery perfusion

#### Preparation of rat pulmonary arterial rings

Rats weighing 250–300 g were anesthetized by intraperitoneal injection of sodium pentobarbital (40 mg/kg), and then their hearts and lungs were removed and immersed immediately in ice-cold KH solution containing (in mmol/L): 118 NaCl, 4.7 KCl, 2.5 CaCl<sub>2</sub>, 1.2 MgSO<sub>4</sub>·7H<sub>2</sub>O, 1.2 KH<sub>2</sub>PO<sub>4</sub>, 25 NaHCO<sub>3</sub>, and 11.1 glucose (pH 7.4). The intrapulmonary arteries (0.7–1.5 mm in diameter) were dissected free of surrounding connective tissue and adventitia and then cut into rings of approximately 2–3 mm in length. The rings were suspended in organ chambers filled with 10 mL of KH solution at 37°C and gassed with 95% O<sub>2</sub>–5% CO<sub>2</sub><sup>[8]</sup>, and isometric tension was measured using a force transducer (JH-2, Space Medico-Engineering Institute, Beijing, China).

#### Vessel ring activity assessment and endothelial function assessment

Vessel rings were allowed to equilibrate for 2 h under a basal tension of 400 mg, during which time the KH solution was changed every 15 min; then 1 μmol/L of NE was added into the chamber to assess the activity of each ring by detecting contraction percentage. Before and after the experimental protocol, the contraction percentage was measured (Figure 2A and 2B). Endothelium was removed in some rings by gently rubbing the intimal surface with a fine steel wire, and the integrity was assessed qualitatively by the degree of relaxation caused by ACh (10 μmol/L) in the percentage of contractile tone induced by NE (1 μmol/L). If the relaxation with ACh was greater than 80%, the endothelium remained in good condition; if the relaxation was less than 30%, the endothelium was destroyed (Figure 2A and 2B)<sup>[9]</sup>.

$$\text{Contraction percentage} = \frac{\text{Tension induced by NE}}{\text{Basal tension}} \times 100\%$$

$$\text{Relaxation percentage} = \frac{\text{Tension induced by vasodilator}}{\text{Tension induced by NE}} \times 100\%$$

#### Isolation and culture of rat PASMCs<sup>[10]</sup>

Distal pulmonary arteries were isolated from pulmonary lobes of rats, and the endothelium and adventitia were removed carefully. After tissues were minced into 2-mm pieces, they were added to the flasks immediately, maintained at 37°C and 5% CO<sub>2</sub> in a humidified incubator for approximately four hours, and then cultured with high-glucose DMEM supplemented with 20% FBS, 100 U/mL of penicillin and 100 U/mL of streptomycin for approximately five days. Half of the culture media was replaced with fresh media when cells emerged. When cells reached 80% confluence, they were harvested with 0.125% trypsin and seeded into the flasks (1:2 ratio) containing high-glucose DMEM supplemented with 10% FBS, 100 U/mL of penicillin and 100 U/mL of streptomycin. Cells with three to eight passages were used for all experiments.

### Identification of PSMCs by immunohistochemistry<sup>[10]</sup>

PASMCs were positive for immunochemical staining when typical "hill and valley" features were observed. Cells were seeded in 6-well plates with glass coverslips. When cells reached 80% confluence, they were fixed in 4% paraformaldehyde, blocked with 3% hydrogen peroxide for 15 min, and then incubated with mouse anti-rat primary  $\alpha$ -SMA antibody overnight at 4°C. After being washed 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.

### Detection of the intracellular calcium concentration in rat PSMCs

PASMCs were seeded in 6-well plates with glass coverslips. When cells reached 80% confluence, the culture media were replaced with serum-free DMEM. Twenty-four hours later, cells were loaded with 7  $\mu$ mol/L Fluo 4-AM at 37°C for 30 min in a humidified atmosphere of 5% CO<sub>2</sub>. The residual dye was washed with Hanks' Balanced Salt Solution (HBSS) solution containing the following (in mmol/L): 1.26 CaCl<sub>2</sub>, 0.49 MgCl<sub>2</sub>·6H<sub>2</sub>O, 0.41 MgSO<sub>4</sub>·7H<sub>2</sub>O, 5.33 KCl, 0.44 KH<sub>2</sub>PO<sub>4</sub>, 4.17 NaHCO<sub>3</sub>, 137.93 NaCl, 0.34 Na<sub>2</sub>HPO<sub>4</sub>, and 5.56 D-Glucose, with no phenol red. Cells loaded with Fluo 4-AM were exposed to one of three treatment conditions: (1) the control group (con group), in which cells were incubated with serum-free DMEM; (2) the NE group, in which cells were incubated with serum-free DMEM supplemented with 1  $\mu$ mol/L of NE for 10 min; and (3) the NE+ECH (100  $\mu$ mol/L) group (NE+ECH<sub>100</sub> group), in which cells were incubated with serum-free DMEM supplemented with 1  $\mu$ mol/L of NE for 10 min and 100  $\mu$ mol/L of ECH for 20 min. Fluorescence intensity was observed and photographed by fluorescence microscopy (IX71, Olympus, Tokyo, Japan)<sup>[11]</sup>. Six fields of vision (200 $\times$ ) were collected for each sample. Intracellular calcium concentration was quantified by measuring mean fluorescence intensity using Image Pro-Plus 6.0 software.

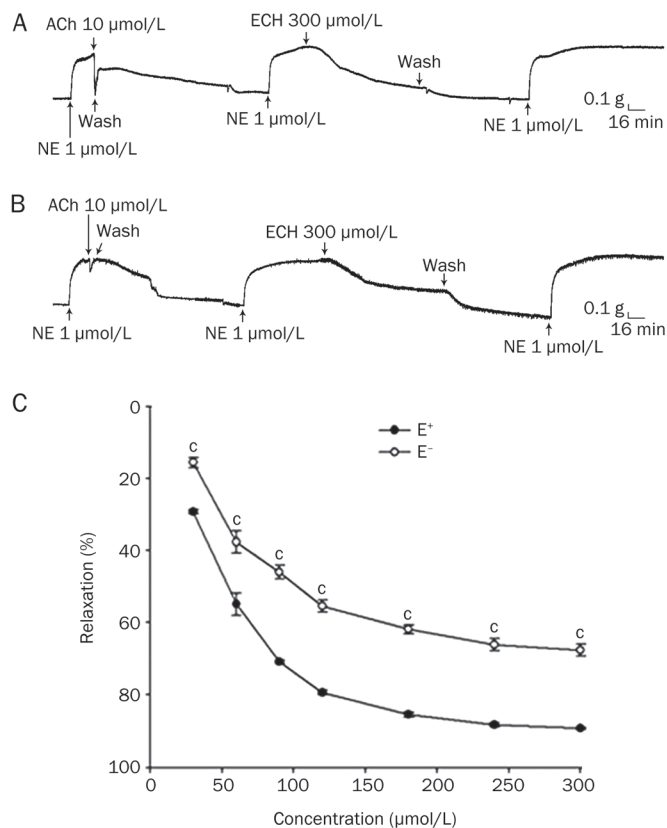
### Statistical analysis

Data were expressed as the mean $\pm$ SD. When appropriate, significant difference was assessed by Dunnett's test or the Student-Newman-Keuls test for multiple comparisons after one-way analysis of variance (ANOVA). A probability level of  $P < 0.05$  was considered significant.

## Results

### Vasorelaxant effects of ECH on isolated pulmonary artery

At the beginning of the experiment, the cumulative addition of ECH from 30 to 300  $\mu$ mol/L had no significant effect on the basal tension of the pulmonary artery. After the NE-induced vasoconstriction reached a plateau, ECH was added cumulatively (30–300  $\mu$ mol/L) to the intact endothelium (E<sup>+</sup>, Figure 2C) or endothelium-denuded rings (E<sup>-</sup>, Figure 2C). In intact endothelium rings, cumulative addition of ECH dilated the vessels in a concentration-dependent manner, with a maxi-



**Figure 2.** The vasorelaxant effects of ECH on E<sup>+</sup> and E<sup>-</sup> pulmonary arterial rings. Representative curve of echinacoside-induced (300  $\mu$ mol/L) relaxant effects on E<sup>+</sup> (A) and E<sup>-</sup> (B) rings ( $n=8$ ); (C) ECH dilated the vessels in a concentration-dependent manner in both E<sup>+</sup> and E<sup>-</sup> pulmonary arterial rings. However, the effect was significantly reduced in the E<sup>-</sup> group ( $^*P < 0.01$  vs E<sup>+</sup>).

imum relaxation percentage of 89.22 $\pm$ 0.32% at 300  $\mu$ mol/L and an EC<sub>50</sub> of 51.60 $\pm$ 0.57  $\mu$ mol/L (Figure 2A and 2C). Similarly, in endothelium-denuded rings, ECH also dilated the vessels in a concentration-dependent manner, but with a significantly lower maximum response of 67.72 $\pm$ 1.69% ( $P < 0.01$  vs intact endothelium rings) and a higher EC<sub>50</sub> of 108.51 $\pm$ 2.8  $\mu$ mol/L ( $P < 0.01$  vs intact endothelium rings, Figure 2B and 2C). The sustained plateau of the high-contraction percentage induced by NE (1  $\mu$ mol/L) after the experimental protocol indicated that the rings pretreated with ECH (30–300  $\mu$ mol/L) had good activity (Figure 2A and 2B).

### Effects of ECH on intracellular calcium release and extracellular calcium influx

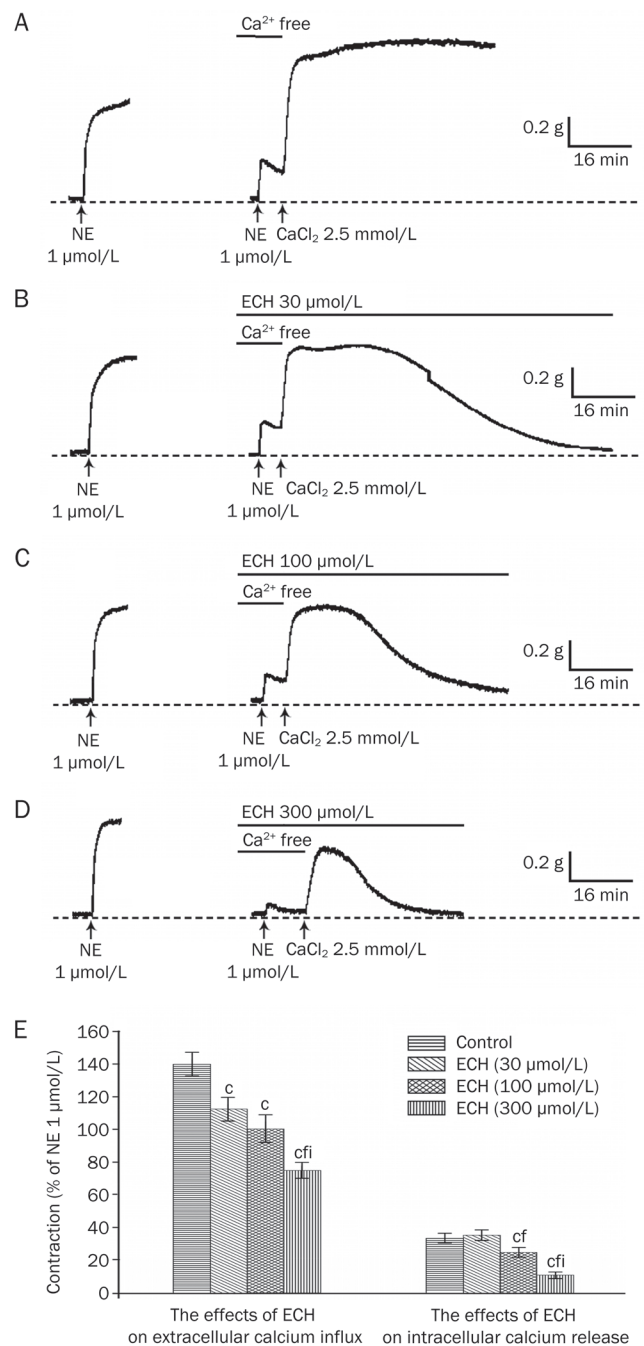
The endothelium-denuded rings were exposed to 1  $\mu$ mol/L of NE until the maximum contraction was attained. Under extracellular Ca<sup>2+</sup>-free conditions, the rings were equilibrated in Ca<sup>2+</sup>-free KH solution containing 0.2 mmol/L of EGTA before the initiation of the experiments. Following three washes with Ca<sup>2+</sup>- and EGTA-free KH solution, the rings were pre-incubated with ECH (30, 100, and 300  $\mu$ mol/L) for 20 min and then re-exposed to 1  $\mu$ mol/L of NE. The rings produced

unsustained contractions that rapidly returned to baseline, compared with the initial contractions induced by NE, which served as the reference. The maximum contraction of the control was  $33.27\pm 2.94\%$  (Figure 3A and 3E) but was significantly reduced to  $24.54\pm 2.97\%$  and  $10.60\pm 2.07\%$  in rings pretreated with 100 and 300  $\mu\text{mol/L}$  of ECH, respectively ( $P<0.01$  vs control group; Figure 3C–3E). Under extracellular calcium influx conditions, 2.5  $\text{mmol/L}$   $\text{CaCl}_2$  was added to the chamber when the curve reached a plateau. The control group produced sustained contractions, with a maximum contraction of  $139.89\pm 7.38\%$  (Figure 3A and 3E); but the maximum contraction was significantly reduced to  $112.42\pm 7.30\%$ ,  $100.29\pm 8.66\%$ , and  $74.74\pm 4.95\%$  in rings pretreated with 30, 100, and 300  $\mu\text{mol/L}$  of ECH, respectively ( $P<0.01$  vs control group; Figure 3B–3E). Under these conditions, the maximal effect caused by ECH was more pronounced with 300  $\mu\text{mol/L}$  than with 100 or 30  $\mu\text{mol/L}$  ( $P<0.01$  vs 30 or 100  $\mu\text{mol/L}$  ECH group). Different concentrations of ECH all shortened the platform of sustained contraction, which was induced by extracellular calcium influx at different levels (Figure 3A–3D).

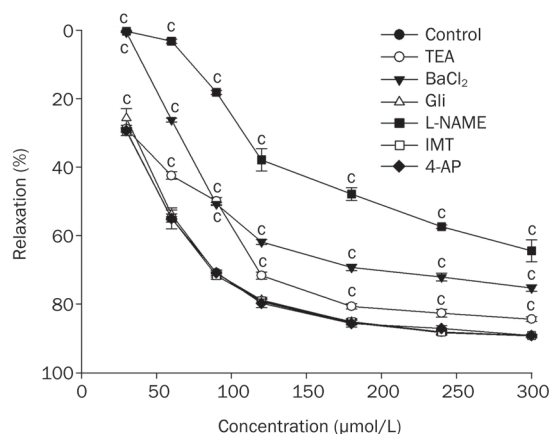
#### Roles of different inhibitors in ECH-induced rat pulmonary artery vasorelaxation

ECH-induced rat pulmonary artery vasorelaxation was investigated in the presence or absence of the following inhibitors: *L*-NAME (eNOS inhibitor), IMT (cyclooxygenase inhibitor), TEA (large-conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channel inhibitor),  $\text{BaCl}_2$  (inward rectifier  $\text{K}^+$  channel inhibitor), 4-AP (voltage-dependent  $\text{K}^+$  channel inhibitor), and Gli (ATP-sensitive  $\text{K}^+$  channel inhibitor). The rings were maximally contracted with 1  $\mu\text{mol/L}$  of NE, and then *L*-NAME (100  $\mu\text{mol/L}$ ), IMT (10  $\mu\text{mol/L}$ ), TEA (1  $\text{mmol/L}$ ),  $\text{BaCl}_2$  (1  $\text{mmol/L}$ ), 4-AP (1  $\text{mmol/L}$ ), or Gli (10  $\mu\text{mol/L}$ ) were added, respectively. Once a new plateau was reached, ECH was added in a cumulative manner from 30 to 300  $\mu\text{mol/L}$ . The concentration–response curves in the presence of different inhibitors are shown in Figure 4. The vasorelaxant effect of ECH on pulmonary arterial rings contracted with 1  $\mu\text{mol/L}$  of NE was significantly blocked by *L*-NAME (100  $\mu\text{mol/L}$ ), TEA (1  $\text{mmol/L}$ ), and  $\text{BaCl}_2$  (1  $\text{mmol/L}$ ), with maximum relaxations of  $64.41\pm 3.20\%$ ,  $84.38\pm 0.70\%$ , and  $75.27\pm 0.93\%$ , respectively ( $P<0.01$  vs the control group of  $89.22\pm 0.32\%$ ), and the values of  $\text{EC}_{50}$  were increased to  $197.32\pm 2.75$   $\mu\text{mol/L}$ ,  $91.42\pm 2.11$   $\mu\text{mol/L}$ , and  $115.49\pm 1.75$   $\mu\text{mol/L}$ , respectively ( $P<0.01$  vs the control group of  $51.60\pm 0.57$   $\mu\text{mol/L}$ ) (Table 1). However, IMT (10  $\mu\text{mol/L}$ ), 4-AP (1  $\text{mmol/L}$ ), and Gli (10  $\mu\text{mol/L}$ ) did not inhibit the ECH-induced relaxation (Figure 4 and Table 1).

To validate the effects of these inhibitors on ECH-induced relaxation, the rings precontracted with 1  $\mu\text{mol/L}$  of NE were treated with *L*-NAME (100  $\mu\text{mol/L}$ ), IMT (10  $\mu\text{mol/L}$ ), TEA (1  $\text{mmol/L}$ ),  $\text{BaCl}_2$  (1  $\text{mmol/L}$ ), 4-AP (1  $\text{mmol/L}$ ), and Gli (10  $\mu\text{mol/L}$ ). After a new plateau was reached, ECH was added to the chamber at a single concentration of 300  $\mu\text{mol/L}$  rather than in a cumulative manner. The maximum relaxation was reduced to  $56.33\pm 0.90\%$ ,  $82.21\pm 0.92\%$ , and  $72.16\pm 0.76\%$  following the addition of *L*-NAME, TEA, and  $\text{BaCl}_2$ , respec-



**Figure 3.** The effects of ECH on intracellular calcium release and extracellular calcium influx. (A) Under extracellular  $\text{Ca}^{2+}$ -free conditions, 1  $\mu\text{mol/L}$  of NE induced short and small contraction (intracellular calcium release), and the addition of 2.5  $\text{mmol/L}$   $\text{CaCl}_2$  resulted in extracellular calcium influx and sustained contraction (control,  $n=8$ ); (B–D) The effects of ECH (30, 100, and 300  $\mu\text{mol/L}$ ) on intracellular calcium release and extracellular calcium ( $n=8$ ). (E) Under extracellular  $\text{Ca}^{2+}$ -free conditions, the maximum contraction was significantly reduced to  $24.54\pm 2.97\%$  and  $10.60\pm 2.07\%$  in rings treated with 100 and 300  $\mu\text{mol/L}$  of ECH, respectively ( $P<0.01$  vs control). Under extracellular calcium influx conditions, it was reduced to  $112.42\pm 7.30\%$ ,  $100.29\pm 8.66\%$ , and  $74.74\pm 4.95\%$  in rings treated with 30, 100, and 300  $\mu\text{mol/L}$  of ECH, respectively ( $P<0.01$  vs control). Additionally, the effect was more pronounced with 300  $\mu\text{mol/L}$  of ECH ( $^cP<0.01$  vs control,  $^fP<0.01$  vs 30  $\mu\text{mol/L}$  ECH,  $^iP<0.01$  vs 100  $\mu\text{mol/L}$  ECH).



**Figure 4.** The roles of different inhibitors in ECH-induced rat pulmonary artery vasorelaxation ( $n=8$ ). The rings were maximally contracted with 1  $\mu\text{mol/L}$  of NE, and then L-NAME (100  $\mu\text{mol/L}$ ), IMT (10  $\mu\text{mol/L}$ ), TEA (1 mmol/L),  $\text{BaCl}_2$  (1 mmol/L), 4-AP (1 mmol/L), or Gli (10  $\mu\text{mol/L}$ ) were added. Once a new plateau was reached, ECH was added in a cumulative manner from 30 to 300  $\mu\text{mol/L}$ . The concentration-response curves in the absence or presence of different inhibitors are shown. The vasorelaxant effect of ECH was significantly blocked by L-NAME (100  $\mu\text{mol/L}$ ), TEA (1 mmol/L), and  $\text{BaCl}_2$  (1 mmol/L), with a maximum relaxation of  $64.41\% \pm 3.20\%$ ,  $84.38\% \pm 0.70\%$ , and  $75.27\% \pm 0.93\%$ , respectively ( $^{\circ}P < 0.01$  vs control group of  $89.22\% \pm 0.32\%$ ), and the concentration-response curves all shifted up. However, IMT (10  $\mu\text{mol/L}$ ), 4-AP (1 mmol/L), and Gli (10  $\mu\text{mol/L}$ ) did not inhibit ECH-induced relaxation.

**Table 1.** The  $\text{EC}_{50}$  of ECH acted on pulmonary artery in the presence or absence of these blockers ( $^{\circ}P < 0.01$  vs control).

Group	$\text{EC}_{50}$ ( $\mu\text{mol/L}$ )
Control	$51.60 \pm 0.57$
L-NAME (100 $\mu\text{mol/L}$ )	$197.32 \pm 2.75^{\circ}$
TEA (1 mmol/L)	$91.42 \pm 2.11^{\circ}$
$\text{BaCl}_2$ (1 mmol/L)	$115.49 \pm 1.75^{\circ}$
Glibenclamide (10 $\mu\text{mol/L}$ )	$54.78 \pm 1.58$
Indomethacin (10 $\mu\text{mol/L}$ )	$51.26 \pm 0.96$
4-AP (1 mmol/L)	$51.47 \pm 0.53$

tively ( $P < 0.01$  vs the control group of  $91.16\% \pm 0.55\%$ ) (Figure 5A, 5B, 5D, 5F, and 5H). However, IMT, 4-AP, and Gli did not inhibit the ECH-induced relaxation (Figure 5A, 5C, 5E, 5G, and 5H).

#### Identification of rat PSMCs

At low magnification, spindle cells were densely packed, and almost all of cells were stained positively for PSMCs (the PSMC purity was  $>95\%$ ) (Figure 6A). Brown-stained myofilaments were clearly observed in the cytoplasm at high magnification (Figure 6B).

#### Effect of ECH on the intracellular calcium concentration in rat PSMCs

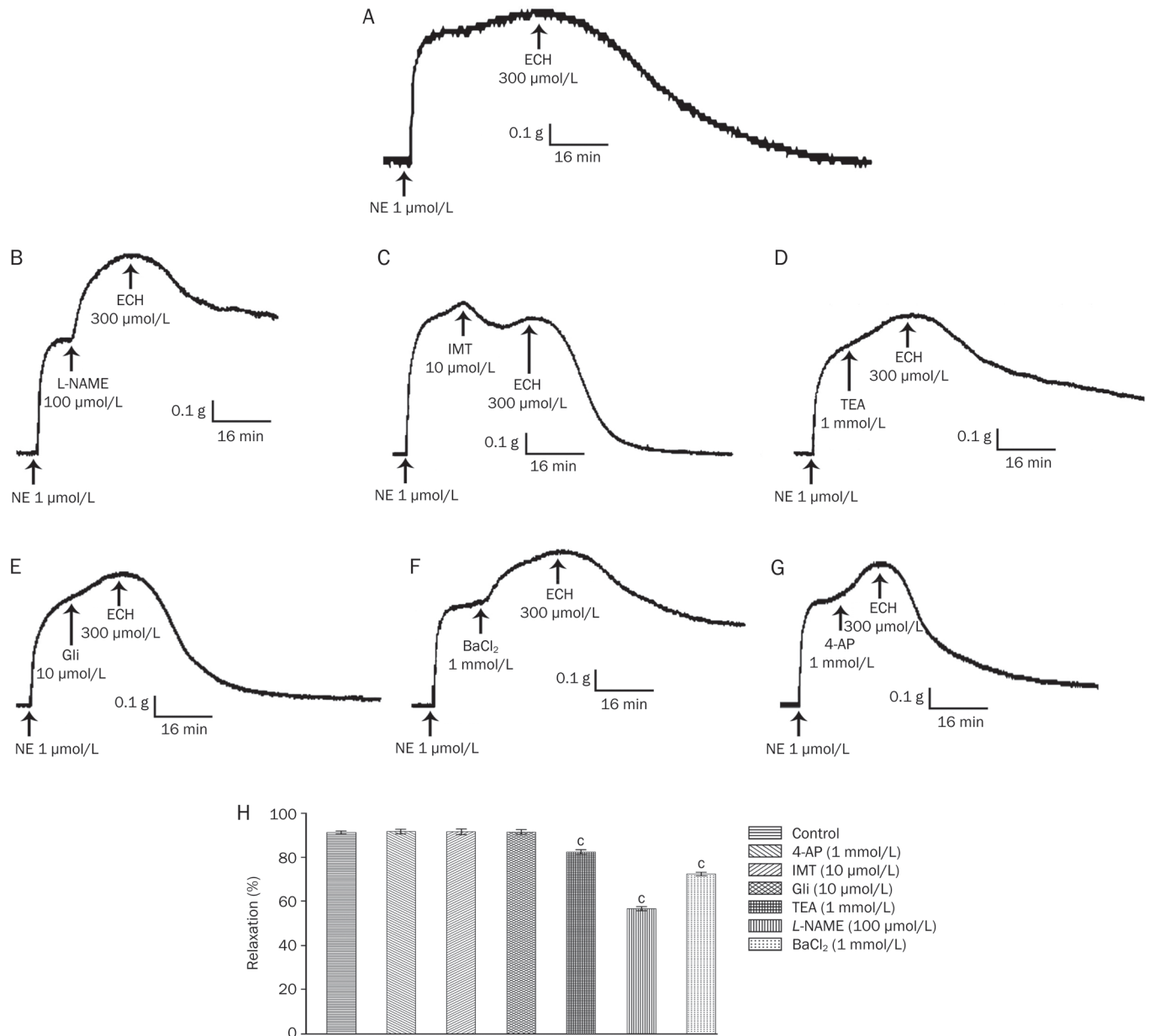
NE (1  $\mu\text{mol/L}$ ) significantly increased the intracellular calcium concentration ( $n=6$ ,  $P < 0.01$  vs the control group) in rat PSMCs, and ECH could decrease the mean fluorescence intensity ( $n=6$ ,  $P < 0.01$  vs the NE group) (Figure 7).

#### Discussion

To the best of our knowledge, this is the first study evaluating the effect of ECH on the vascular tone of the rat pulmonary artery. We found that ECH induced vasorelaxation of rat pulmonary artery precontracted with NE in a concentration-dependent manner, and such effects can be observed in both intact endothelium and endothelium-denuded rings. In the pulmonary artery, ECH attenuated both extracellular calcium influx and intracellular calcium release, and the NO-cGMP pathway and the opening of  $\text{K}^+$  channels (large conductance  $\text{Ca}^{2+}$ -activated  $\text{K}^+$  channels and inward rectifier  $\text{K}^+$  channels) appeared to play a role in ECH-induced rat pulmonary artery vasorelaxation. In rat PSMCs, ECH could reduce intracellular calcium concentration directly. Previous studies have also suggested that ECH could mediate the endothelium-dependent vasodilator action in rat thoracic aortic rings through the NO-cGMP pathway<sup>[7]</sup>.

ECH-induced vasorelaxation can be observed in both intact endothelium and endothelium-denuded rings. However, removal of endothelium resulted in a significant reduction in maximum response and an increase in the  $\text{EC}_{50}$  relative to the intact endothelium rings. It has been suggested that NO-cGMP and  $\text{PGI}_2$ -cAMP pathways play a key role in regulating endothelium-dependent vascular relaxation<sup>[9]</sup>. To explore this issue, we added 100  $\mu\text{mol/L}$  of L-NAME, an inhibitor of NO synthesis into endothelial cells. The results showed that L-NAME significantly attenuated the vasodilation of ECH, suggesting that NO is likely to play a role in the vasorelaxation of ECH. We also found that IMT, an inhibitor of cyclooxygenase (a key  $\text{PGI}_2$  synthetase), had no such effect, indicating that  $\text{PGI}_2$  was unlikely to be involved in the vasorelaxation of ECH. Therefore, ECH may enhance NO production from L-arginine in vascular endothelium and activated guanylate cyclase, thus catalyzing the conversion of GTP to cGMP. After the experimental protocol, the rings showed the sustained plateau of high contraction percentage induced by NE (1  $\mu\text{mol/L}$ ), which indicated that the rings pretreated with ECH (30 to 300  $\mu\text{mol/L}$ ) displayed good activity (Figure 2A and 2B).

Vascular tone is regulated by intracellular  $\text{Ca}^{2+}$ <sup>[12]</sup>. We found that ECH (100 and 300  $\mu\text{mol/L}$ ) significantly reduced the phasic contraction induced by NE in endothelium-denuded pulmonary arterial rings in  $\text{Ca}^{2+}$ -free KH solution, and this effect was more pronounced with the higher concentration of ECH (300  $\mu\text{mol/L}$ ), indicating that the inhibitory effect of ECH on the NE-induced contraction of pulmonary arterial rings was mediated by interference in the ability of  $\text{IP}_3$  to promote the release of intracellular  $\text{Ca}^{2+}$ . NE acts on the  $\alpha$ -receptor to mediate vascular contraction, thus increasing intracellular



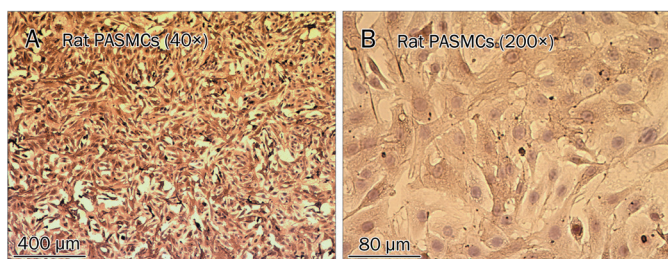
**Figure 5.** The effects of different inhibitors on ECH (300  $\mu\text{mol/L}$ )-induced rat pulmonary artery vasorelaxation ( $n=8$ ). (A) Control group ECH (300  $\mu\text{mol/L}$ ), (B) L-NAME (100  $\mu\text{mol/L}$ ), (C) IMT (10  $\mu\text{mol/L}$ ), (D) TEA (1 mmol/L), (E) Gli (10  $\mu\text{mol/L}$ ), (F)  $\text{BaCl}_2$  (1 mmol/L), or (G) 4-AP (1 mmol/L) was applied to the rings precontracted with 1  $\mu\text{mol/L}$  of NE (control). (H) The maximum relaxation was reduced to 82.21%±0.92%, 56.33%±0.90%, and 72.16%±0.76% following the addition of TEA, L-NAME, and  $\text{BaCl}_2$ , respectively ( $^cP < 0.01$  vs the control). However, IMT, 4-AP, and Gli did not inhibit ECH-induced relaxation.

$\text{Ca}^{2+}$  levels ( $[\text{Ca}^{2+}]_i$ ) through the G-protein coupled phosphatidylinositol signal transduction pathway<sup>[13]</sup>. Once being activated, this triggers the formation of the second messenger IP<sub>3</sub> and diacylglycerol induced by phospholipase C<sup>[14]</sup>. IP<sub>3</sub> can rapidly mobilize and release  $\text{Ca}^{2+}$  from the sarcoplasmic reticulum to cause a transient increase in  $[\text{Ca}^{2+}]_i$ , which could be associated with the NE-induced phasic contraction in pulmonary arterial rings in  $\text{Ca}^{2+}$ -free medium.

To investigate the effect of ECH on the extracellular calcium influx,  $\text{CaCl}_2$  (2.5 mmol/L) was added under  $\text{Ca}^{2+}$ -free

conditions. The addition of ECH (30, 100, and 300  $\mu\text{mol/L}$ ) significantly reduced the maximum contraction, with the most significant effect observed with 300  $\mu\text{mol/L}$  ECH. This suggested that ECH inhibits intracellular calcium release and extracellular calcium influx in PASMCS. However, further studies are needed to elucidate the relationship between ECH and inhibitors of L-type calcium channels.

To investigate the role of calcium, Fluo 4-AM was used to determine intracellular calcium concentrations in rat PASMCS. Because the addition of NE (1  $\mu\text{mol/L}$ ) resulted in a rapid



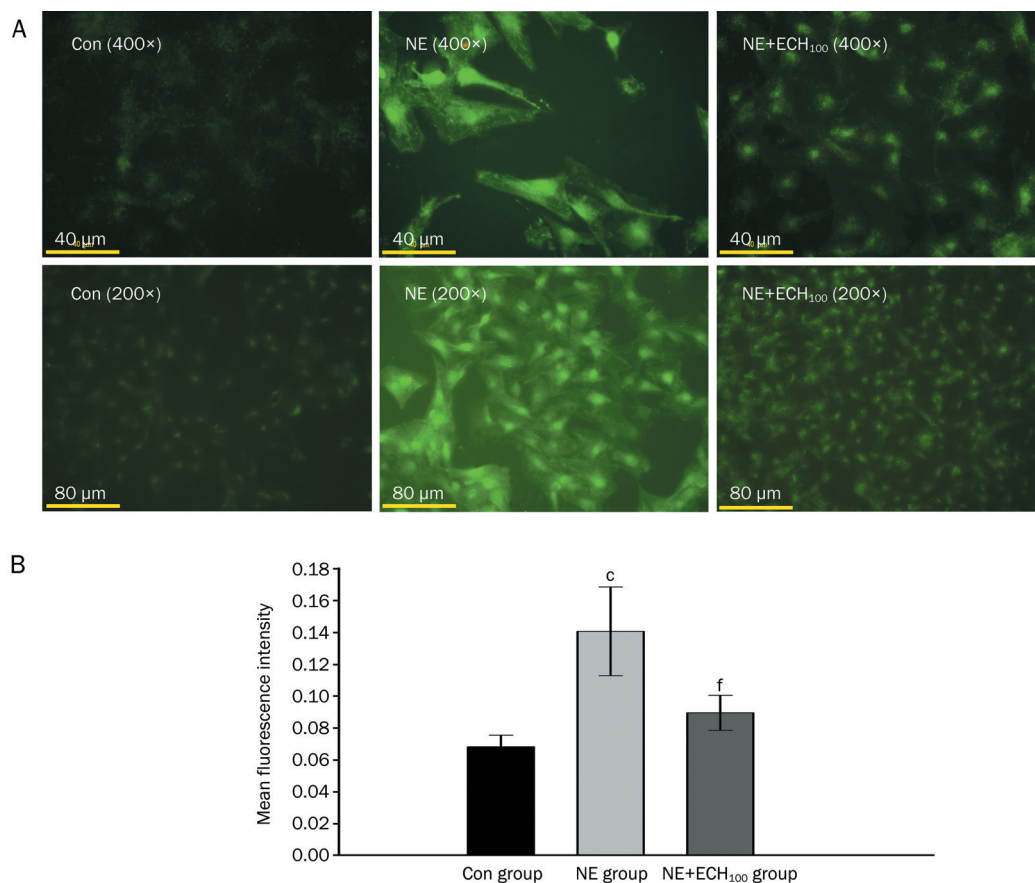
**Figure 6.** Identification of PSMCs by immunohistochemistry. (A) At low magnification, spindle cells were densely packed, almost all of cells were positive for PSMCs (the PSMC purity was >95%). (B) Brown-stained myofilaments were clearly observed in the cytoplasm at high magnification.

change in the tension of vessel rings (Figure 2), rat PSMCs were first loaded with Fluo 4-AM, and then NE and ECH were added. The results showed that 1  $\mu\text{mol/L}$  NE could increase the intracellular calcium concentration, which was in agreement with previous studies, and the mean fluorescence intensity was lower in the NE+ECH<sub>100</sub> group than in the NE group. Thus, it was concluded that ECH could reduce the intracel-

lular calcium concentration in rat PSMCs pretreated with NE. Because it is preferable to detect intracellular calcium concentration in a real-time manner, further research is being conducted to observe the real-time changes of intracellular calcium concentration using confocal microscopy.

K<sup>+</sup> channels contribute to the regulation of pulmonary vascular tone through cytoplasmic K<sup>+</sup> and Ca<sup>2+</sup> concentration and membrane potential. The opening of K<sup>+</sup> channels leads to an efflux of K<sup>+</sup> that causes the cell membrane to become hyperpolarized, leading to the closure of L-type calcium channels and the reduction of intracellular Ca<sup>2+</sup> [15, 16]. In this study, we found that BaCl<sub>2</sub> (an inhibitor of K<sub>IR</sub> channels) and TEA (an inhibitor of BK<sub>Ca</sub> channels) remarkably reduced the vasorelaxant effect of ECH, indicating that the K<sub>IR</sub> and BK<sub>Ca</sub> channels could be involved in this effect. However, 4-AP (an inhibitor of K<sub>v</sub> channels) and Gli (a K<sub>ATP</sub> inhibitor) had no such effect, indicating that the K<sub>v</sub> or K<sub>ATP</sub> channels were not involved.

BK<sub>Ca</sub> channels, with seven transmembrane domains and a calcium-binding region (bowl), are somewhat structurally unique, even within the K<sub>Ca</sub> family [17, 18]. They are expressed widely in smooth muscle cells and play an important role in regulating vascular tone, which is activated by both membrane depolarization and intracellular calcium [19-21]. BK<sub>Ca</sub>



**Figure 7.** The effect of ECH on the intracellular calcium concentration in rat PSMCs. (A) The image of intracellular calcium fluorescence (200 $\times$  and 400 $\times$ ). (B) NE (1  $\mu\text{mol/L}$ ) significantly increased the intracellular calcium concentration ( $n=6$ ,  $^{\circ}P<0.01$  vs the control group) in rat PSMCs, and ECH could decrease the mean fluorescence intensity ( $n=6$ ,  $^{\circ}P<0.01$  vs the NE group).

channels have also been proposed as potential therapeutic targets for cardiovascular diseases because of their ability to regulate vascular tone<sup>[22, 23]</sup>. We found that TEA, an inhibitor of BK<sub>Ca</sub> channels, caused the concentration-response curve to shift up and a significant increase in the EC<sub>50</sub>. We also found that the ECH-induced relaxation was significantly weaker at the highest concentration of ECH in rings after treatment with TEA (1 mmol/L) compared with the control. NO-induced vasodilation is due in part to a cGMP-dependent, protein kinase G-mediated activation of BK<sub>Ca</sub> channels<sup>[24]</sup>. Therefore, we assumed that ECH induced rat pulmonary artery vasorelaxation via the NO-cGMP-PKG-BK<sub>Ca</sub> channels, which led to a decrease in intracellular Ca<sup>2+</sup> levels and the relaxation of PSMCs. An interesting observation was that different concentrations of ECH reduced the maximum contraction induced by extracellular calcium influx and shortened the sustained contraction, which may be related to the opening of BK<sub>Ca</sub> channels.

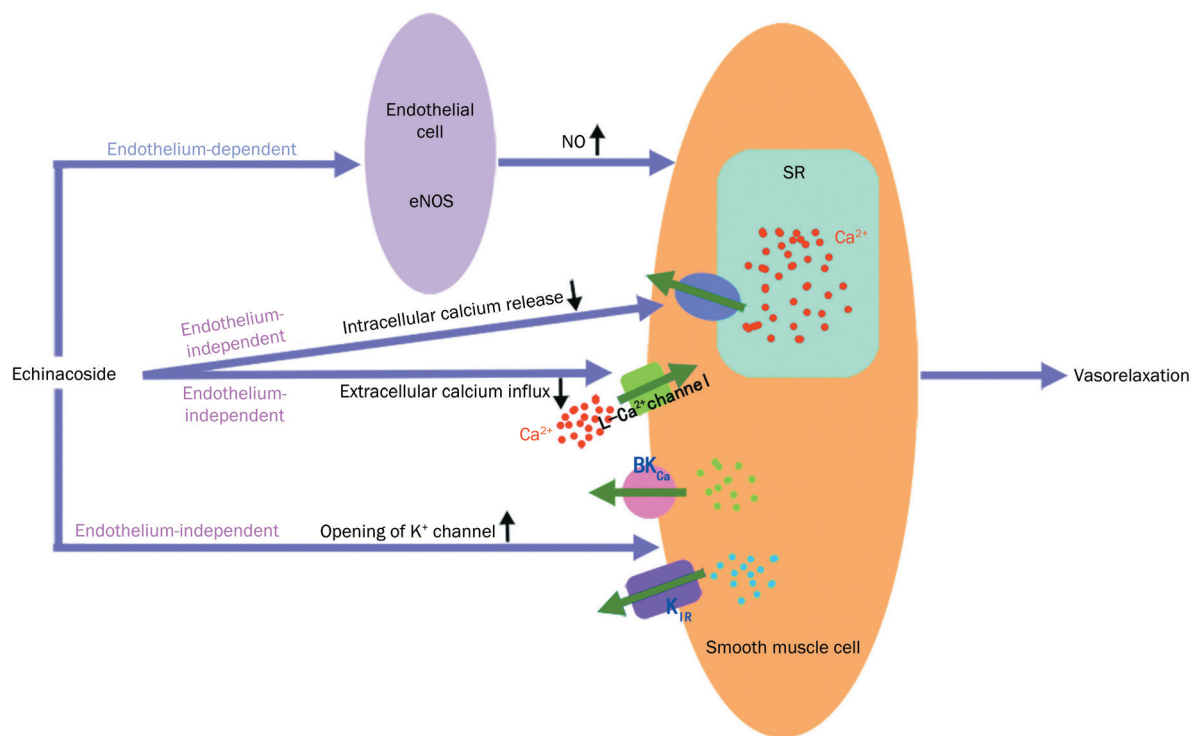
K<sub>IR</sub> channels are the simplest channels, with only two transmembrane domains. They are involved in regulating the driving force for calcium entry in pulmonary vascular endothelial cells; therefore, they have been implicated in regulating vascular permeability<sup>[25]</sup> and responses to shear stress<sup>[26]</sup>. In this study, we showed that the K<sub>IR</sub> inhibitor caused the concentration-response curve to shift up and a significant increase in EC<sub>50</sub>, and that ECH (300 μmol/L)-induced relaxation was signifi-

cantly weaker compared with the control. All these results indicated that ECH had a vasorelaxant effect on the pulmonary artery via the K<sub>IR</sub> channels. However, given the complex role of K<sup>+</sup> channels in regulating vascular tone, further studies are required to elucidate this issue. K<sup>+</sup> channels have recently been suggested to participate in vascular remodeling by regulating cell proliferation and apoptosis<sup>[18]</sup>. Global hypoxia, as it occurs at high altitude, causes pulmonary vasoconstriction or vascular remodeling and increases pulmonary vascular resistance<sup>[27]</sup>. Impaired K<sup>+</sup> channels (K<sub>ATP</sub>, K<sub>Ca</sub>, K<sub>v</sub>, and K<sub>IR</sub>) in vascular smooth muscle cells are also related to the development of hypertension<sup>[22, 28]</sup>.

Hypoxic inhibition of K<sup>+</sup> channels results in membrane depolarization, the activation of voltage-dependent Ca<sup>2+</sup> (Ca<sub>v</sub>) channels, extracellular Ca<sup>2+</sup> influx and intracellular Ca<sup>2+</sup> release, thus resulting in an increase in [Ca<sup>2+</sup>]<sub>i</sub>, followed by pulmonary vasoconstriction and proliferation of PSMCs<sup>[29]</sup>. Opening K<sup>+</sup> channels contributes to the regulation of hypoxic pulmonary vasoconstriction and vascular remodeling. Thus, ECH may open the K<sup>+</sup> channels, thereby attenuating hypoxic pulmonary vasoconstriction as well as reducing vascular remodeling.

## Conclusion

ECH induces vasorelaxation in rat pulmonary arteries precontracted with NE, and such an effect can be observed in both



**Figure 8.** Graphical abstract. ECH induced vasorelaxation of rat pulmonary arteries precontracted with NE, and this effect could be observed in both intact endothelium and endothelium-denuded rings. In PSMCs, ECH attenuated both extracellular calcium influx and intracellular calcium release, which resulted in vasoconstriction; additionally, the NO-cGMP pathway and the opening of K<sup>+</sup> channels (BK<sub>Ca</sub> and K<sub>IR</sub>) appeared to play a major role in ECH-induced rat pulmonary artery vasorelaxation.



intact endothelium and endothelium-denuded rings. This is most likely related to the opening of the NO-cGMP-PKG-BK<sub>Ca</sub> channels and the reduction of intracellular Ca<sup>2+</sup> levels, thereby relaxing the PSMCs (Figure 8).

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### Author contribution

Ri-li GE and Xiang-yun GAI designed the study; Xiang-yun GAI, Yu-hai WEI, Ta-na WUREN, Ya-ping WANG, Zhan-qiang LI, and Yi ZHOU performed the research; Wei ZHANG and Yu-hai WEI contributed new reagents and analytical tools; Xiang-yun GAI and Shou LIU analyzed the data; Xiang-yun GAI wrote the paper; and Lan MA and Dian-xiang LU modified the paper.

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