Original Research

Effects of electro-acupuncture on endothelium-derived endothelin-1 and endothelial nitric oxide synthase of rats with hypoxia-induced pulmonary hypertension

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Abstract

Pulmonary hypertension (PH) is characterized by elevated pulmonary artery pressure (PAP), pulmonary vascular remodeling and right ventricular hypertrophy, which are mainly due to endothelial dysfunction. Electro-acupuncture has shown beneficial effects on cardiovascular homeostasis, but little evidence has been obtained on pulmonary effects. The goal of the present study was to investigate whether electro-acupuncture on bladder-13 and -15 points can protect against chronic hypoxia-induced PH by regulating endothelium-derived endothelin (ET)-1 and endothelial nitric oxide synthase (eNOS). Male Wistar rats were exposed to hypoxia to induce PH. Hemodynamic analysis revealed that mean PAP was similar under normoxic conditions. Chronic hypoxia increased mean PAP to 37 $\pm$ 3 mmHg, and electro-acupuncture attenuated it to 29 $\pm$ 3 mmHg. Absolute right ventricular weight was ameliorated by electro-acupuncture from 0.288 $\pm$ 0.048 g to 0.228 $\pm$ 0.029 g under hypoxic conditions. Hypoxia-induced right ventricular hypertrophy index decreased from 0.477 $\pm$ 0.069 to 0.378 $\pm$ 0.053 with electro-acupuncture treatment. Histological examination revealed that hypoxic rats showed increased medial pulmonary artery wall thickness as well as muscularization. However, these alternations by chronic hypoxia were attenuated by electro-acupuncture. There was no difference in eNOS or ET-1 between groups under normoxic conditions. Electro-acupuncture treatment significantly improved the circulating eNOS concentration (365.36 $\pm$ 31.51 pg/mL) compared with only hypoxia exposure (247.60 $\pm$ 30.64 pg/mL). In lung homogenate, levels of eNOS under hypoxia increased from 684.96 $\pm$ 117.90 to 869.86 $\pm$ 197.61 pg/mg by electro-acupuncture treatment. Levels of ET-1 changed oppositely to eNOS in response to electro-acupuncture (ET-1 in plasma, 29.44 $\pm$ 2.09 versus 20.70 $\pm$ 2.37 pg/mL; ET-1 in lung homogenate, 120.51 $\pm$ 3.03 versus 110.60 $\pm$ 4.04 pg/mg). In conclusion, these results indicated that treatment with electro-acupuncture can protect against hypoxia-induced PH, possibly by regulating the balance of endothelium-derived vasoconstrictors and vasodilators.

Keywords: hypoxia, pulmonary hypertension, electro-acupuncture, endothelial nitric oxide synthase, endothelin-1

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Introduction

Pulmonary hypertension (PH), a fatal complication of chronic lung disease and heart failure, is characterized by elevated pulmonary artery pressure (PAP), pulmonary vascular remodeling and right ventricular hypertrophy.[1–3] Although its pathogenesis is not yet fully understood, endothelial dysfunction has been thought to have a pivotal role in PH under hypoxic conditions. Endothelial dysfunction not only results in excessive pulmonary vascular constriction, but also leads to disorganized pulmonary arterial smooth muscular cell proliferation and distal extensions into small non-muscular pulmonary arteries[4,5] with the physiological balance between vasodilators and vasoconstrictors shifted towards the latter.[6] Increasing evidence has demonstrated that improving endothelial dysfunction and/or restoring the altered balance of endothelium-derived mediators has beneficial effects on PH.[4,7]

Acupuncture treatment has become a novel complementary therapy and alternative conventional medicine in clinical practice because of safety and lack of untoward reactions.[8,9] Mounting evidence has revealed the curative role of acupuncture in cardiovascular homeostasis, especially combined with modern electrical techniques.[10,11] The underlying mechanism of vascular tone regulated by acupuncture is incompletely understood. However, effects of reducing sympathetic nervous system activation, inhibiting the production of

proinflammatory cytokines and regulating endothelium homeostasis have been taken into account. Regulating the homeostasis of endothelium-derived mediators is thought to play a key role in its therapeutic effect.[12,13]

In this study, the effect of electro-acupuncture treatment on hypoxia-induced PH was investigated. Furthermore, to gain insight into the underlying mechanism, we investigated whether the effects were associated with the balance between endothelium-derived endothelin (ET)-1 and endothelial nitric oxide synthase (eNOS).

Materials and methods

Experiment groups and animal models

All animal care and experimental protocols were in accordance with the guide for the Animal Care and Use of the Ministry of Health of the People's Republic of China (documentation number 19890503) and the Animal Management Rules of Harbin Medical University (China).

Forty Wistar rats (220–240 g) were involved in this experiment and randomly divided into four groups ($n = 10$ each): a normoxic group (N group), a normoxic and electro-acupuncture treatment group (N-EA group), a hypoxic group (H group) and a hypoxic and electro-acupuncture treatment group (H-EA group). Rats were exposed to hypoxia (9–11%) to progressively develop PH according to the method published by Zhu *et al.*[14] Briefly, rats were raised in a normobaric and hypoxic chamber (150 cm × 70 cm × 60 cm) for 21 d to induce PH. The oxygen concentration in the chamber was maintained at 9–11% by controlling the inflow rate of nitrogen and measured daily (DATEX AS/3, Datex, Finland). Carbon dioxide was abolished by sodalime granules and the redundant were discharged through the holes on the chamber. Excess humidity and ammonia were prevented by anhydrous calcium chloride and boric acid. The chamber was opened for 1 h daily to clean the cage, replenish food and water and perform the electro-acupuncture treatment. Rat chow and water were available *ad libitum*.

Electro-acupuncture treatment

Electro-acupuncture stimulation was provided at two pairs of acupoints, bladder-13 (BL-13) and bladder-15 (BL-15), in accordance with the traditional Chinese medical theory and the principle of selecting nearby acupoints. BL-13 and BL-15 are located paravertebral, which are 1 cm horizontally from the third and fifth thoracic vertebra, respectively. Hwato needles (diameter, 0.25 mm; length, 30 mm; Suzhou Medical Appliance Factory, Suzhou, China) were inserted into the four acupoints at a depth of about 6 mm perpendicularly, and electric stimulation was provided through the needles by an electro-acupuncture treatment device (Great wall KWD-808, Hangzhou, China). The output rarefaction wave was kept at 1 V with a frequency of 5 Hz. Rats in the N-EA and H-EA groups received electro-acupuncture treatment daily from the 10th day to the 14th day and from the 17th day to the 21st day for a period of 30 min under normoxic conditions. The time course of hypoxia and treatment were reminded by an electrical calendar. A registered acupuncturist blind to group assignments performed the treatment.

Hemodynamic measurements

After 21 d of hypoxic exposure, rats received 60 mg/kg sodium pentobarbital intraperitoneally. After tracheotomy was performed, the lungs were mechanically ventilated (NatureGene Corp., Harvard 683, South Natick, MA, USA) with room air at 60 beats/min. Mean artery pressure (MAP) was measured by a catheter in the left femoral artery and heart rate (HR) was calculated by an electrocardiagraph. A catheter attached to a pressure transducer was inserted into the main pulmonary artery via the right ventricular outflow tract after the midsternal thoracotomy,[15] and mean PAP was continuously recorded (Powerlab/16SP, AD Instruments, Castle Hill, NSW, Australia) in the anesthetized rats for a 2-min recording.

Tissue preparation and right ventricular hypertrophy analysis

After measurement of hemodynamics, a blood sample was drawn from the left ventricle (LV) and centrifuged at 2000*g* for 15 min. Plasma was stored at $-80\,^{\circ}C$ for subsequent measurements. The heart and lung were harvested *en bloc*. A transverse lung slice of the left lung was embedded in paraffin for morphological examination, while the others were stored at $-80\,^{\circ}C$. The heart was dissected free from the atria, aorta and pulmonary trunk. The right ventricle (RV) was separated from the LV and ventricular septum (S). RV and LV plus S were weighed separately to determine absolute RV weight and RV to LV + S ratio (RV/(LV + S)).

Lung morphological measurement

Lung tissues embedded in paraffin were cut into 3-μm sections that were stained with elastic stains, as well as immunohistochemistry with anti-α-smooth muscle actin.

Pulmonary vascular hypertrophy was assessed by percent medial wall thickness (%MT) of the pulmonary vasculature. It was expressed as the medial wall thickness divided by the diameter of the vessel. The %MT was calculated for at least 10 pulmonary arteries for each rat.

Pulmonary arterial muscularization, estimated through immunohistochemistry with anti-α-smooth muscle actin (Abcam, Cambridge, MA, USA), was determined by the ratio of vessels in each category to the total number counted. Muscularization was divided into three categories as described previously[16]: non-muscularized, partially muscularized and fully muscularized.

All morphological analyses were performed by two independent observers blind to the experimental design. Inter-observer difference in the measurements was $<5\%$.

Assay for ET-1 and eNOS levels

Endothelium-derived eNOS and ET-1 of plasma and lung homogenate were measured with a sandwich enzyme-linked immunosorbent assay as described in commercial kits (R&D Systems, Minneapolis, MN, USA).

Immunoblotting of ET-1 protein

Lung homogenate was suspended in RIPA lysis buffer including a cocktail of protease inhibitors (Roche, West Sussex, UK) and centrifuged at 8000*g* for 5 min. Then, the supernatant was loaded (50 μg) and separated on a 12% sodium dodecyl sulfate-polyacrylamide gel followed by blotting of the proteins onto the polyvinylidene difluoride membrane (Bio-Rad Laboratories, Hercules, CA, USA). After blocking non-specific binding with non-fat dry milk for four hours at room temperature, membranes were incubated with mouse ET-1 monoclonal antibody (dilution, 1: 300; Santa Cruz Biotechnology, Heidelberg, Germany) for two hours at room temperature, then incubated with secondary anti-mouse antibody conjugated with horseradish peroxidase for one hour. Proteins were visualized by chemiluminescence. All gels were repeated in quadruplicate. The levels of the measured proteins were normalized to the level of actin.

Statistical analysis

Values were expressed as means $\pm$ SD. Statistical analysis of data was performed by one-way analysis of variance (ANOVA) followed by least significant difference (LSD) analysis or two-way ANOVA using group and treatment as independent factors as appropriate. A value of $P < 0.05$ was considered statistically significant.

Results

Systemic and pulmonary hemodynamic analyses

There was no difference in systemic hemodynamic data between groups. MAP did not differ among the groups (N group, 119 ± 7 mmHg; N-EA group, 114 ± 8 mmHg; H group, 115 ± 10 mmHg; and H-EA group, 107 ± 12 mmHg; $P > 0.05$). Although electro-acupuncture did not influence HR, chronic hypoxia increased HR from 392 ± 2 beats/min to 402 ± 2 beats/min ($P < 0.05$).

The mean PAP of rats under hypoxic conditions was 37 ± 3 mmHg, nearly two times as high as that of rats under normoxic conditions (N group, 16 ± 2 mmHg; N-EA group, 17 ± 3 mmHg, $P < 0.05$). In contrast, electro-acupuncture treatment resulted in a marked decrease in mean PAP of the H-EA group (29 ± 3 mmHg, $P < 0.05$) (Figure 1A).

Right ventricular hypertrophy analysis

There were no significant differences in absolute RV weight and RV/(LV + S) of rats under normoxic conditions (absolute RV weight: N group, 0.181 ± 0.033 g versus N-EA group, 0.152 ± 0.023 g; RV/(LV + S): N group, 0.259 ± 0.044 versus N-EA group, 0.231 ± 0.045; $P > 0.05$). Hypoxia elicited greater absolute RV weight and RV/(LV + S) than normoxia, and electro-acupuncture attenuated those changes induced by hypoxia exposure (absolute RV weight: 0.288 ± 0.048 g versus 0.228 ± 0.029 g; RV/(LV + S): 0.477 ± 0.069 versus 0.378 ± 0.053; $P < 0.05$) (Figures 1B and C).

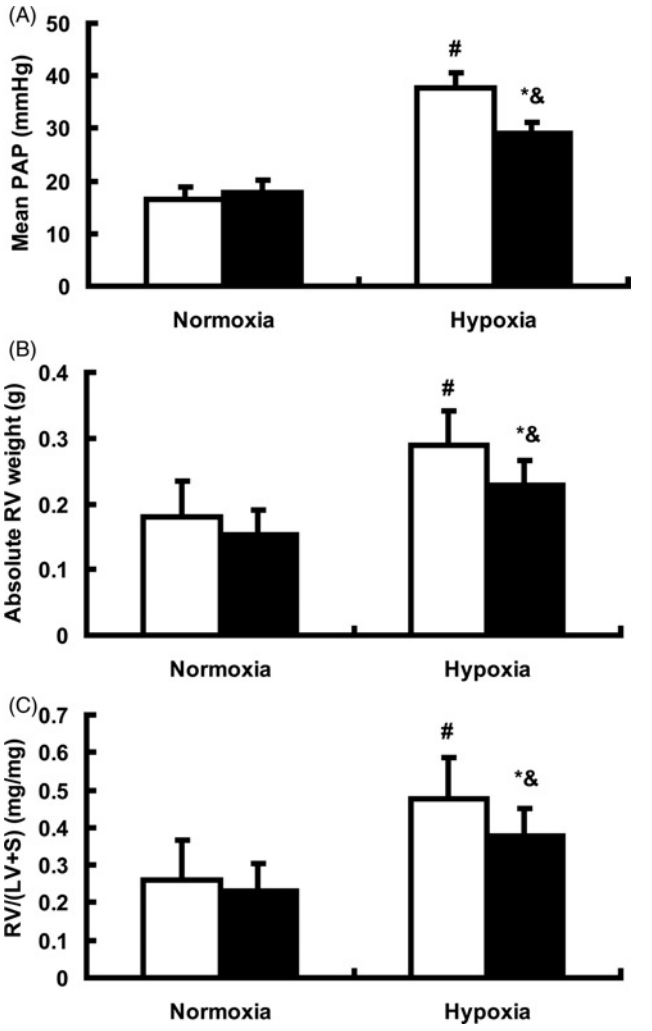

Figure 1 Effects of electro-acupuncture on pulmonary hemodynamics and right ventricular hypertrophy. (A) Effects of electro-acupuncture on mean PAP, (B) absolute RV weight and (C) the ratio of right ventricular weight to left ventricular plus septum weight (RV/(LV + S)). Open bars = rats without electro-acupuncture treatment, solid bars = rats with electro-acupuncture treatment. The data are mean $\pm$ SD ($n = 10$ each). $^{\#}P < 0.05$ versus normoxic rats, $^{*}P < 0.05$ versus hypoxic rats without electro-acupuncture, $^{\&}P < 0.05$ versus normoxic rats. PAP, pulmonary artery pressure, RV, right ventricle, LV, left ventricle, S, ventricular septum

Morphometric analysis

The distribution of muscularized arteries dramatically increased under hypoxic exposure (Figures 2A and B). The percentage of muscularized arteries was significantly higher in the H group than in the H-EA group ($P < 0.05$) (Figure 2C). As shown in Figure 2D, the medial wall thickness of pulmonary vessels was also augmented by chronic hypoxia. However, medial wall thickness was lower in the H-EA group than in the H group ($P < 0.05$).

eNOS levels of plasma and lung homogenate

Figure 3 shows levels of eNOS in circulation and in lung homogenate. No significant difference was observed among the four groups in baseline eNOS levels in plasma or lung homogenate ($P > 0.05$). After 21 d of hypoxic

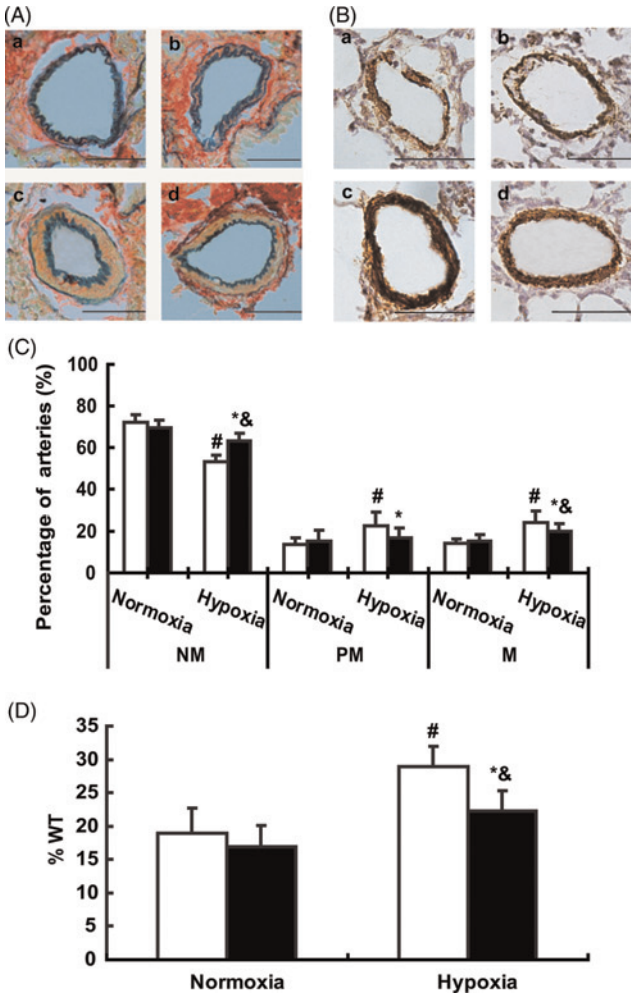

Figure 2 Effect of electro-acupuncture on vascular remodeling. (A) Photomicrographs of peripheral pulmonary arteries on Day 21 with elastic stains. Scale bars = 50 μm. (B) Photomicrographs of immunohistochemical staining for α-smooth muscle actin. Scale bars = 50 μm. (C) The percentage of muscularized arteries. (D) Quantitative analysis of the % WT in peripheral pulmonary arteries. % WT = percent medial wall thickness. a = rats under normoxic conditions, b = rats under normoxic conditions with electro-acupuncture, c = rats under hypoxic conditions, d = rats under hypoxic conditions with electro-acupuncture; open bars = rats without electro-acupuncture treatment, solid bars = rats with electro-acupuncture treatment. The data are mean ± SD ($n = 10$ each). $^{\#}P < 0.05$ versus normoxic rats, $^{*}P < 0.05$ versus hypoxic rats without electro-acupuncture, $^{\&}P < 0.05$ versus normoxic rats. (A color version of the figure is available in the online journal)

exposure, these levels were two times lower than they were under normoxic conditions ($P < 0.05$). However, electro-acupuncture attenuated the reduced levels in the H group.

ET-1 protein expression and levels in plasma and lung homogenate

Pulmonary ET-1 expression was comparable between the N group and N-EA group. Its expression was enhanced by hypoxic exposure, and this was ameliorated by treatment with electro-acupuncture (Figures 4A and B).

The circulating ET-1 concentration was very low at the basal level but increased during chronic hypoxia exposure. After repeated electro-acupuncture treatment, a significantly lower concentration of ET-1 was found in the H-EA group in comparison with the H group (H, 29.44 ± 2.09 pg/mL versus H-EA, 20.70 ± 2.37 pg/mL, $P < 0.05$) (Figure 4C).

The ET-1 concentration of lung homogenate was 103.80 ± 3.06 pg/mg in the N group and 102.68 ± 6.42 pg/mg in the N-EA group, with no statistical difference ($P > 0.05$). Chronic hypoxia elevated ET-1 levels (120.51 ± 3.03 pg/mg), but the effect of hypoxia was attenuated by electro-acupuncture to 110.60 ± 4.04 pg/mg (Figure 4D).

Discussion

The main finding of this study is that electro-acupuncture attenuates certain consequences of PH, including elevated PAP, remodeling of the pulmonary vasculature and right ventricular hypertrophy. Furthermore, preliminary data suggest that electro-acupuncture may exert its beneficial effects by regulating the balance of endothelium-derived eNOS and ET-1.

It has been well recognized that elevated PAP, pulmonary vascular remodeling and right ventricular hypertrophy are characteristics of PH.[2,17] In the present study, the mean PAP of rats exposed to hypoxia almost doubled in comparison with that of normoxic exposure. The right ventricular hypertrophy index, expressed as the ratio of RV to LV plus septum, also increased significantly. The same was true of pulmonary artery muscularization, as well as percent medial wall thickness, which are indications of pulmonary vascular remodeling. Known characteristics of PH induced by chronic hypoxia were all prominently observed in this study. Therefore, the model used in our study was appropriate for evaluating the effects of electro-acupuncture on hypoxia-induced PH.

Electro-acupuncture is one of the techniques of acupuncture within traditional Chinese medical practice. It has a beneficial effect when treating many diseases, such as addiction, pain management and cardiovascular disease.[10,11,18] However, previous studies mainly focused on the effects of electro-acupuncture on cardiovascular homeostasis or systemic hypertension. There has been little research on its pulmonary effects, and to our knowledge, no study has focused on PH. Accumulating evidence shows that nitric oxide (NO) is one of the important signaling molecules in the meridian system, particularly in electro-acupuncture.[19–22] Recent studies have also indicated that ET-1 may be involved in the underlying mechanism of electro-acupuncture.[23–25] At the same time, there is a balance between endothelium-derived vasodilators and vasoconstrictors; this is to say, NO is countered by endothelium-derived ET-1 in the pulmonary vascular system.[7,15,26,27] Therefore, the two mediators were measured simultaneously in our study. The meridian theory, which is an important component treatment system in traditional Chinese medicine, guides the diagnosis and treatment of traditional Chinese medicine in many respects, especially in relation to acupuncture. According to traditional Chinese medical theory, together with the principle of selecting nearby acupoints, BL-13 and BL-15 were selected in our study.

NO derived from endothelium is a potent vasodilator and inhibitor of vascular smooth muscle cell proliferation, which is necessary to maintain pulmonary vascular tone

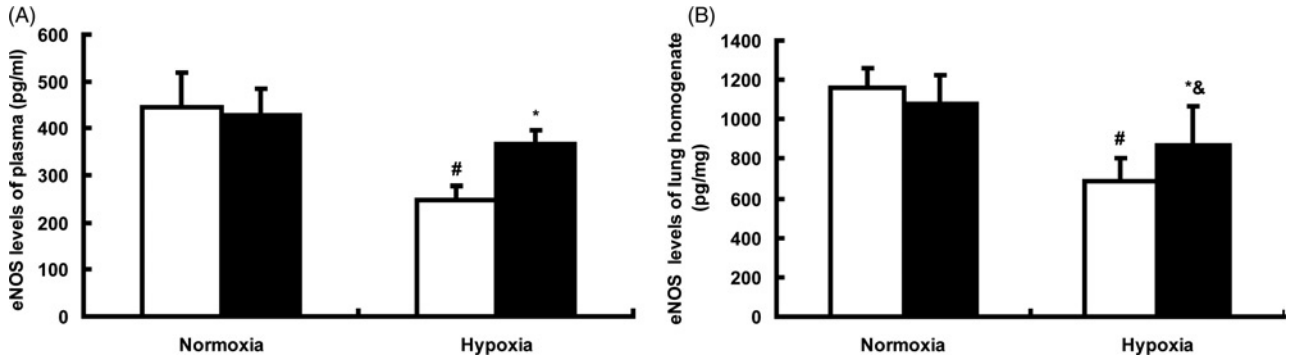

Figure 3 eNOS levels in plasma and lung homogenate. (A) eNOS levels in plasma. (B) eNOS levels in lung homogenate. Open bars = rats without electro-acupuncture treatment, solid bars = rats with electro-acupuncture treatment. The data are mean ± SD ($n = 10$ each). $^{\#}P < 0.05$ versus normoxic rats, $^{*}P < 0.05$ versus hypoxic rats without electro-acupuncture, $^{\&}P < 0.05$ versus normoxic rats. eNOS, endothelial nitric oxide synthase

homeostasis. Similarly, investigations have revealed that levels of eNOS decrease in pulmonary tissue with PH.[28] Furthermore, eNOS-deficient mice showed mild PH.[29] Therefore, the reduced production of eNOS-derived NO causes PH and aggravates pathophysiological progress. These studies also support our results that the basal levels of eNOS were reduced in rats exposed to hypoxia.

The current findings indicate that electro-acupuncture regulates the reduced levels of eNOS. Acupuncture stimulation could reduce sympathetic nervous system activation via the cholinergic system. Huang *et al.*[30] reported that electro-acupuncture can modulate the NOS system in the central nervous system in stress-induced hypertension. In spontaneously hypertensive rats, Kim *et al.*[31] also found similar results. Needling of acupuncture points could also influence muscle afferents, which have stimulating effects on NO local release.[32] Our data are in agreement with the above findings that electro-acupuncture attenuated the reduced eNOS levels. However, we did not evaluate which was more pronounced between central effects or peripheral actions evoked by electro-stimulation. We also did not evaluate the time course correlation between levels of eNOS, development of PH and electro-acupuncture. However, our results clearly demonstrate that electro-acupuncture on BL-13 and BL-15 served to upregulate the reduced levels of eNOS and protect against hypoxia-induced PH.

ET-1, with potent vasoconstrictive activity and mitogenic effects, is widely distributed in pulmonary endothelium.

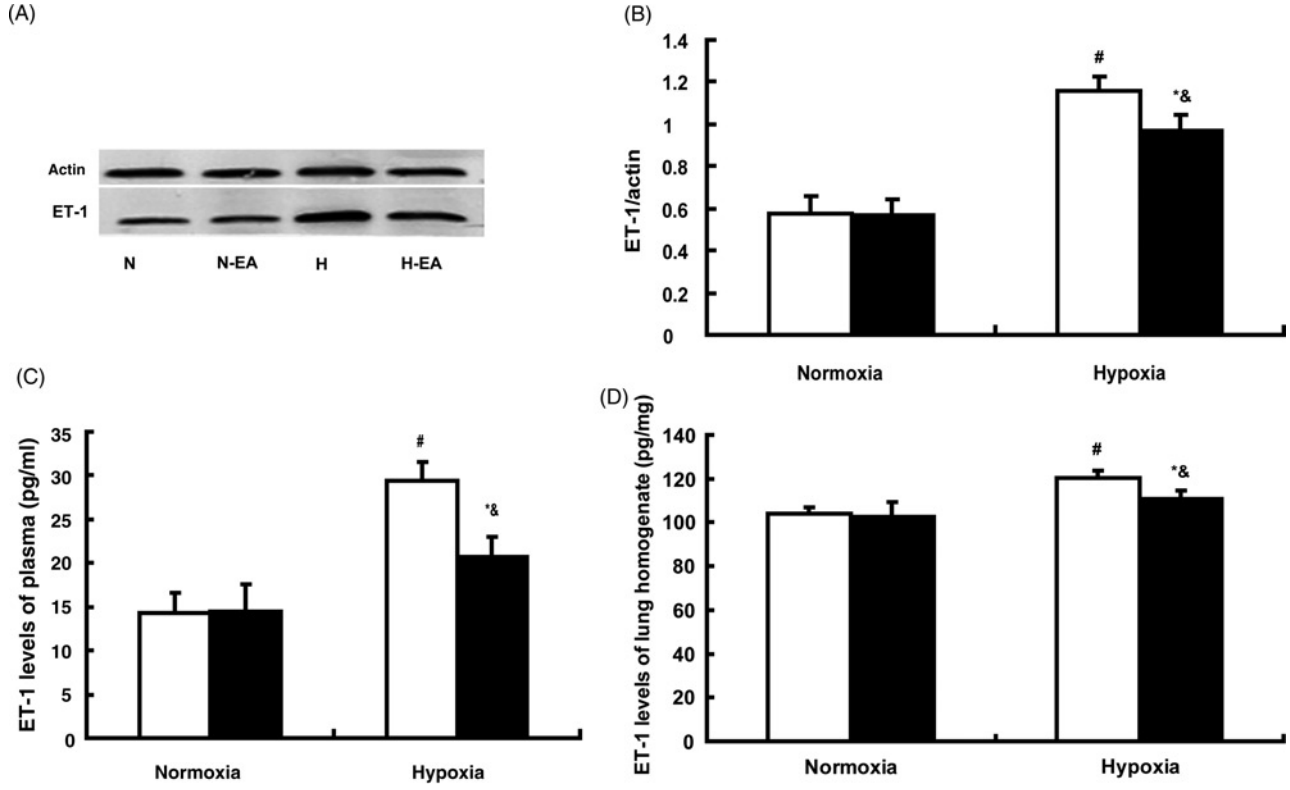

Figure 4 ET-1 levels in plasma and lung homogenate. (A) and (B) ET-1 protein expression in lung homogenate by immunoblotting. (C) ET-1 levels in plasma. (D) ET-1 levels in lung homogenate. Open bars = rats without electro-acupuncture treatment, solid bars = rats with electro-acupuncture treatment. The data are mean ± SD ($n = 10$ each). $^{\#}P < 0.05$ versus normoxic rats, $^{*}P < 0.05$ versus hypoxic rats without electro-acupuncture, $^{\&}P < 0.05$ versus normoxic rats

Mounting evidence has revealed excessive ET-1 expression and release and endothelial dysfunction due to hypoxia insult.[33–35] In the present study, the level of circulating ET-1 under hypoxic conditions was significantly higher than under normoxic conditions, which is in accordance with previous reports.[3,35,36] When the elevation of ET-1 was blocked during chronic hypoxia, as observed by electro-acupuncture treatment in the present study, the increase in mean PAP, pulmonary vascular remodeling and right ventricular hypertrophy were all significantly ameliorated. These observations further support the pivotal role of ET-1 in the development of PH, and together with previous evidence,[12,13,23,24,37] they suggest that the ability of acupuncture to regulate vascular tone is linked to ET-1. The lung is the primary site for ET-1 production,[38] so we also measured ET-1 levels in lung homogenates. Compared with untreated rats under hypoxic conditions, it was dramatically attenuated in rats treated with electro-acupuncture. Chronic hypoxia stimulates excessive ET-1 production and persistent elevation of ET-1 contributes to pulmonary vascular remodeling. In the morphological analysis, the current study demonstrated that increased medial wall thickness and vascular muscularization were attenuated by electro-acupuncture, and this was also confirmed quantitatively. The morphological change was also accompanied by immunoblotting of ET-1 protein. These observations strongly suggested that downregulating increased ET-1 levels contributed to the improvement of hypoxia-induced PH by electro-acupuncture.

Acupuncture has been used therapeutically in China for thousands of years, and it has steadily claimed its usefulness in complementary medicine with its safety and wide acceptance.[39] The effectiveness of acupuncture and electro-acupuncture on vascular tone has been well investigated in animal models[40,41] as well as in clinical practice.[23] In this study, it is worth noting that electro-acupuncture lowered pulmonary arterial pressure in chronic hypoxic pulmonary hypertensive rats but it did not influence MAP in normoxic rats.

A limitation of this observational study is that we measured the balance between vasoconstrictors and vasodilators to investigate the mechanism of electro-acupuncture without taking proliferative/apoptotic signaling pathways into account. Levels of ET-1 and eNOS may not fully explain the underlying mechanism. However, the results indicate that electro-acupuncture is an attractive alternative method of regulating the homeostasis of endothelium-derived mediators.

In conclusion, this study revealed that electro-acupuncture treatment can protect against hypoxia-induced PH. Electro-acupuncture attenuated multiple known consequences of PH in adult chronically hypoxic rats, including elevated mean PAP, right ventricular hypertrophy and pulmonary vascular remodeling. We also found that compared with rats under hypoxic conditions, electro-acupuncture treatment upregulated reduced levels of eNOS and concomitantly downregulated elevated levels of ET-1. Our results may provide a better understanding of meridian theory and an alternative or complementary method when discussing therapeutic strategies for chronic hypoxia PH. Additional studies are also advisable to investigate whether electro-acupuncture treatment can decrease the severity and/or reverse the progression of the disease once PH is established, which is the ultimate clinical relevance of a new therapeutic paradigm.

Author contributions: PP and XZ (co-first authors), HQ, WS, JW, YB and WL (other authors) conceived and designed the research, acquired the data, analyzed the data, performed statistical analysis, handled funding and supervision, drafted the manuscript and made critical revision of the manuscript for important intellectual content. All authors have read and approved the submission of the manuscript to *Experimental Biology and Medicine*. PP and XZ contributed equally to this work.

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Conflict of interest: There was no conflict of interest in this study.

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