

## Effect of thoracic epidural blockade on hypoxia-induced pulmonary arterial hypertension in rats

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### ABSTRACT

**Objective(s):** The present study was aimed to investigate the influence of thoracic epidural blockade on hypoxia-induced pulmonary hypertension in rats.

**Materials and Methods:** Forty eight Wistar rats were randomly divided into 4 equal groups, named normoxia hypoxia hypoxia/ ropivacaine and hypoxia/saline. Animals were placed in a hypoxia chamber and instrumented with epidural catheters at the thoracic level. Rats were injected with saline or ropivacaine. Haemodynamic measurements included pulmonary artery pressure and right ventricular hypertrophy. Degree of pulmonary vascular remodeling was determined by Hematoxylin and Eosin (HE) staining. Serum cyclic GMP (cGMP) and TNF- $\alpha$  were measured using radioimmuno assay. Real-time PCR and western blotting were employed to examine the expression of cAMP responding-element binding protein (CREB).

**Results:** We found that the thoracic epidural blockade significantly decreased chronic hypoxia-induced pulmonary hypertension and vascular remodeling in rats. Ropivacaine-treated rats exhibited significantly lower mean pulmonary artery pressure (mPAP), ratio of right ventricular weight to left ventricular plus septal weight (RV/(LV+S)) and wall thickness of pulmonary artery compared with those of control rats. Hypoxia-induced increase in levels of serum cGMP and TNF- $\alpha$  was reversed by thoracic epidural blockade. Moreover, hypoxia increased expression of CREB at mRNA and protein levels which could be suppressed by thoracic epidural blockade.

**Conclusion:** Thoracic epidural blockade reduced mPAP and serum level of TNF- $\alpha$  and increased cGMP. The treatment reversed upregulated expression of CREB at mRNA and protein production.

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### Introduction

Pulmonary arterial hypertension (PAH) is characterized by profound increase in pulmonary vascular resistance in association with a degree of vascular proliferation and remodeling, vasoconstriction, and in situ thrombosis. The disorder progressively leads to right heart failure and high mortality. The cellular and molecular mechanisms underlying the development of PAH are investigated intensively and continued to develop new treatment. Current therapies for PAH include anticoagulation, lung transplantation, atrial septostomy, and pulmonary endarterectomy (1, 2). Therapeutic agents under investigation include calcium-channel blockers, prostacyclin analogues, endothelin-1 receptor antagonists, phosphodiesterase inhibitors and L-arginine (3-5). In addition, experimental treatments including genetic therapy, stem cell therapy, and anti-proliferative therapies are of interest for the management of PAH (6).

Hypoxia is one of typically frequent causative

factors inducing PAH, leading to pulmonary vasoconstriction and vascular remodeling (7). Hypoxia-associated vascular remodeling is attributed to aberrant proliferation of endothelial cells, smooth muscle cells and adventitial fibroblasts, resulting in increased resistance in the pulmonary circulation accompanying right ventricular failure (8). Additionally, hypoxia exposure leads to sympathetic nervous system (SNS) activation which acts as a compensating mechanism to assure oxygen supply for critical organs (9, 10, 13, 14). It has been reported that chronic hypoxia increased systemic arterial pressure and massive activation of the SNS (11, 12). It is suggested that enhanced SNS activity in response to hypoxia plays a critical role in pathogenesis of hypertension.

Thoracic epidural blockade (TEB) is a standard procedure that is commonly exercised for pain management after a variety of surgeries. In addition to brilliant analgesic properties, it has been shown to

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have effects far beyond pain management (15). TEB has been demonstrated to reduce post-operative pulmonary complications, and to attenuate the peri-operative stress response (16, 17). It is evident that TEB constitutes cardioprotective effect through cardiac sympathetic denervation (18-20). It is suggested that TEB is beneficial to PAH in a hypoxic setting. Current understanding of the effect of TEB on PAH-changes in cardiophysiology such as haemodynamic parameters- is resulted from limited animal models and clinical studies. However, the molecular mechanism underlying the effect of TEB on vasculature in hypoxia-induced PAH setting remains vague. Additionally, inflammatory responses to have been considered to contribute to the pathogenesis of PAH. It is of interest to elucidate the role of inflammation in PAH pathogenesis and the effect of TEB on this disorder.

In this regard, we hypothesized that TEB may ameliorate pulmonary arterial hypertension and vascular remodeling induced by chronic hypoxia.

## Materials and Methods

### Animal and experimental design

Male Wistar rats (obtained from China medical University, China) weighing 150 ~ 190 grams were used. All experimental protocols were reviewed and approved by the animal care committee. Animal were randomly assigned to one of 4 groups: (1) Normoxia: rats exposed to ambient air with no treatment (n=12); (2) Hypoxia: rats exposed to hypoxia with no treatment (n=12); (3) Hypoxia/ ropivacaine: rats exposed to hypoxia and administered with ropivacaine, (n=12); (4) Hypoxia/saline: rats exposed to hypoxia and administered with saline, (n=12).

Hypoxia exposure was conducted as previously described (2). In brief, animals were either exposed to ambient air or placed in a tightly sealed hypoxia chamber for 21 days. Oxygen concentration was maintained at 10% and the ratio of O<sub>2</sub> to CO<sub>2</sub> in chamber was monitored daily.

### Assessment of pulmonary artery pressure

The mean pulmonary artery pressure (mPAP) was measured as described previously (2). Briefly, after 21 days normoxic or hypoxic exposure, the animals were anesthetized with intraperitoneal injection of pentobarbital sodium (10 mg/kg). mPAP was measured through introducing a catheter passed through the right external jugular vein and right ventricle into pulmonary artery. After measurement of mPAP, the animals were sacrificed and used immediately for further experiments including evaluation of right ventricular hypertrophy, immunoblotting and RT-PCR.

### Measurement of right ventricular hypertrophy

The ventricles and septum were harvested, weighed and dehydrated for 24 hr at 60°C. Then a ratio of right

ventricle to left ventricle plus septum weight (RV/(LV+S)) was calculated for determination of right ventricular hypertrophy (2).

### Measurement of pulmonary vascular remodeling

The thickness of pulmonary arteries was measured as previously described (2). Pulmonary arteries were harvested and stained for assessment of medial wall thickness. The percentage of medial wall thickness of pulmonary arteries was used to determine pulmonary artery remodeling. The percentage of wall thickness was calculated as average diameter of the external elastic lamina minus the average diameter of internal elastic lamina divided by the average diameter of external elastic lamina.

### RT-PCR

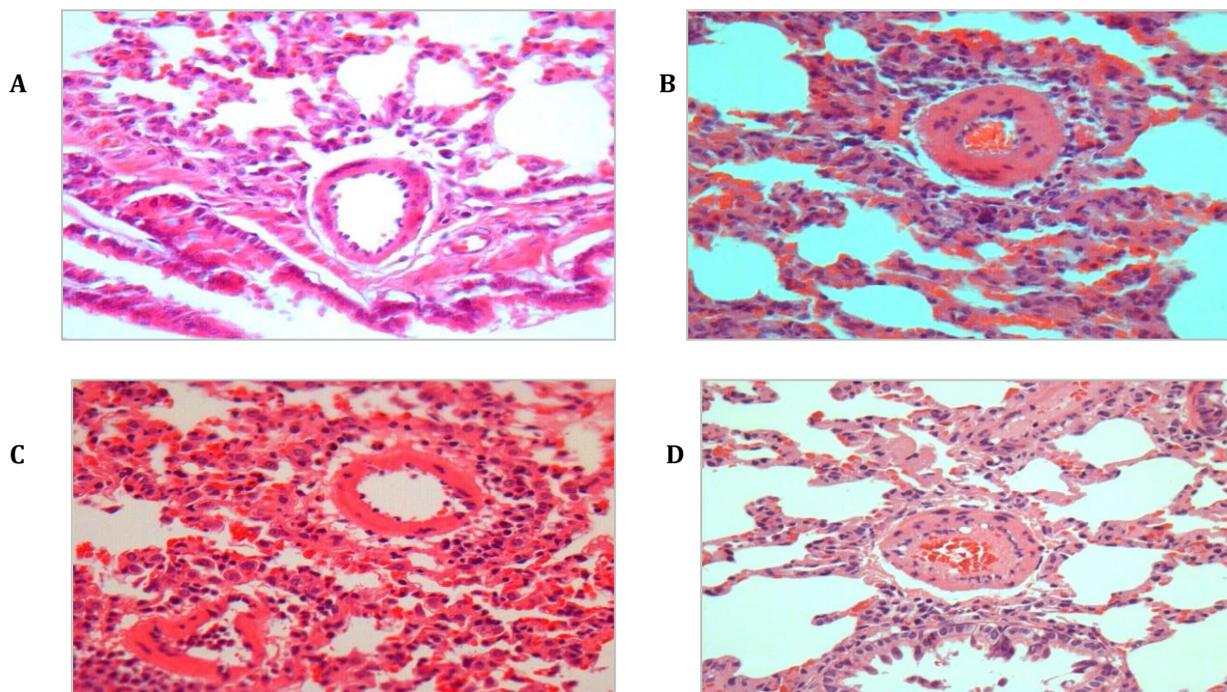
Total RNA was extracted using TRIzol reagent (Ambion, CA). The amount of each RNA sample was determined by Qubit fluorometer (Invitrogen, CA). Reverse transcription was performed in a 20 µl reaction system with 200 ng total RNA using high capacity cDNA reverse transcription kits. Relative quantification of inflammation mediators and apoptosis indicators were assessed by real-time PCR using ABI 7900HT (ABI, CA) system. A housekeeping gene β-actin was used as an internal control. Sequences of primers used were β-actin : 5'-GTCAGGTCATCACTA TCGGCAAT-3' and 5'- AGAGGTCTTTACGGATGTCAACG-3' ; CREB: 5'-TTGTT GTTCAAGCTGCCTCTG-3' and 5'-ACGAACCTCTCTTTCGTGC-3'.

### Western blotting

Tissue samples were harvested and lysed using RIPA buffer (Sigma-Aldrich, CA). Samples were centrifuged at 12000 rpm for 15 min at 4°C. The supernatants were collected to perform immunoblotting. Thirty microgram of protein samples were subjected to 10% SDS-PAGE, then transferred to PVDF (Millipore, MA) for 1 hr at 100 V and 4°C. Membranes were blocked with 5% BSA in TBST and subsequently incubated with rabbit anti-CREB and β-actin (Santa Cruz Biotechnology, CA) for 1 hr at room temperature. The blots were washed by TBST and incubated with secondary antibody for 30 min at room temperature. The blots were assessed using ECL system (Amersham, UK).

### Statistical analysis

All quantitative data were expressed as mean±SD. Statistical analysis was performed using Prism software package (GraphPad v3). One-way ANOVA was conducted to compare each parameter and *Post hoc t* test comparisons were performed to identify which group differences accounted for significant overall ANOVA results. Results were considered significant at *P*<0.05.



**Figure 1.** Effects of TEB on chronic hypoxia-induced pulmonary vascular structure remodeling of rats. Hematoxylin and Eosin staining of pulmonary arterioles. (A) Normoxia (B) Hypoxia (C) Hypoxia/ ropivacaine (D) Hypoxia/ saline

## Results

### TEB decreased hypoxia-induced pulmonary artery pressure and right ventricular hypertrophy in rats

After 3 weeks of hypoxia exposure, rats exhibited pulmonary hypertension characteristics, showing a significant increase in (mPAP) comparing with that of the normoxia group (Table 1,  $P < 0.05$ ). In the hypoxia/ropivacaine group, the pulmonary artery pressure was significantly decreased compared with that of hypoxia/saline group ( $P < 0.05$ ) and their difference with, normoxia group was negligible. There was no

significant difference in the mPAP between hypoxia and hypoxia/saline groups. TEB also significantly ameliorated right ventricular hypertrophy, showing a decrease in the ratio of RV/(LV+S) in the hypoxia/ropivacaine group compared with the hypoxic ones (Table 1).

Vascular remodeling was induced in hypoxic animals, showing an increase in medial wall thickness of pulmonary arteries as compared with the normoxia group. The percentage of wall thickness (WT%) of arterioles, which is the index of pulmonary artery

**Table 1.** Comparison of hemodynamic data and right ventricle hypertrophy index between 4 groups

	Normoxia	Hypoxia	Hypoxia/ ropivacaine	Hypoxia/ saline	P
pH	7.324±0.034	7.305±0.086	7.315±0.055	7.308±0.074	n.s.
PaO <sub>2</sub> (mmHg)	90.94±7.35	49.16±11.58	87.58±8.05	51.04±10.95	<0.05
PaCO <sub>2</sub> (mmHg)	41.55±6.32	43.21±7.25	42.01±7.09	43.52±6.98	n.s.
HR (beats/min)	447±21	428±11	439±14	430±12	n.s.
mSAP (mmHg)	96±4	101±3	98±5	102±4	n.s.
CO (ml/min)	142±18	102±15	136±21	105±14	<0.05
mPAP (mmHg)	17.00±1.84	29.75±2.79	20.50±2.07	27.85±3.13	<0.05
RV/(LV+S)	19.90±1.39	29.25±2.42	20.00±1.42	29.40±2.63	<0.05
WA %	59.67±3.20	76.58±2.98	61.08±5.75	79.53±4.71	<0.05
WT %	4.12±0.20	8.00±0.55	4.36±0.34	7.68±0.53	<0.05

All values are expressed as mean ± SD. PaO<sub>2</sub>: arterial pressure of O<sub>2</sub>; PaCO<sub>2</sub>: arterial pressure of CO<sub>2</sub>; HR: heart rate; mSAP: mean systemic arterial pressure; CO: cardiac output; mPAP: mean pulmonary arterial pressure; RV/(LV+S): right ventricular hypertrophy index; WA%: wall area ratio; WT%: wall thickness ratio. n.s.: not significant

**Table 2.** Effect of TEB on serum levels of cyclic GMP and TNF-α in hypoxia-exposed rats

	Normoxia	Hypoxia	Hypoxia/ ropivacaine	Hypoxia/ saline	P
cGMP (pmol/ml)	5.05±1.06	2.75±0.85	5.10±0.76	3.25±0.58	<0.005
TNF-α (pmol/ml)	0.613±0.137	1.184±0.283	0.585±0.120	0.923±0.213	<0.005

All values are expressed as mean ± SD (n = 12); cGMP: cyclic GMP; TNF-α: tumor necrosis factor-alpha

remodeling, was significantly increased in response to chronic hypoxia exposure in comparison with the normoxia group (Table 1). Treatment of rats with the ropivacaine significantly ameliorated the increment of the wall thickness of pulmonary arteries induced by hypoxia. There was no significant difference in the WT% between hypoxia and hypoxia/saline groups. The microscopic results revealed the incidence of smooth muscle cell proliferation and hypertrophy, and inflammatory cell infiltration (Figure 1). In accordance with the wall thickness, the wall area percentage (WA%) in the hypoxia group was also significantly higher than in the normoxia group (Table 1,  $P < 0.05$ ).

#### TEB modulated serum levels of cyclic GMP and TNF- $\alpha$

It is documented that hypoxic pulmonary hypertension is associated with decreased cyclic GMP (cGMP) level and an elevated production of TNF- $\alpha$  in serum (3, 4). In consistency with previous studies, chronic hypoxia caused a significant decrease in cGMP level and a 2-fold increase in TNF- $\alpha$  serum level compared with those of normoxia group (Table 2). The decreased level of cGMP in hypoxia-exposed rat was significantly ameliorated by administration of ropivacaine, whereas cGMP level was unaffected in hypoxia/ saline group. Our results show that TEB significantly ameliorated hypoxia-mediated elevation of TNF- $\alpha$  level from  $1.184 \pm 0.283$  to  $0.585 \pm 0.120$  pmol/ml (Table 2). There was no difference in TNF- $\alpha$  level in the group of hypoxia/ saline.

#### The effect of TEB on cAMP responding-element binding protein (CREB)

##### Hypoxia

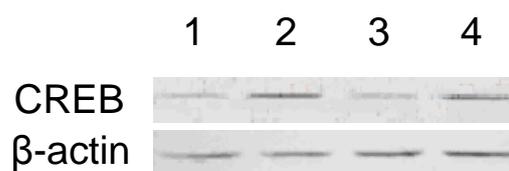
The expression of CREB in lung tissue of hypoxia-exposed rats was determined in transcriotnal and translational levels. Using RT-PCR, mRNA expression of CREB was upregulated in lung tissue of rats exposed to chronic hypoxia (Table 3). Treatment of hypoxia-exposed rats with TEB resulted in a significant suppression of CREB expression in lung tissue at mRNA level. Consistently, protein level of CREB in lung tissue of hypoxia-exposed rats was significantly reduced by TEB compared with that of hypoxia/saline group (Figure 2).

## Discussion

Pulmonary arterial hypertension is commonly complicated with many lung diseases such as cardiac obstructive pulmonary disease (COPD). It is evident

that the presence of chronic hypoxia is also associated with PAH, which plays a critical role in the pathogenesis of PAH with respect to myocardial dystrophy. Chronic hypoxia leads to excessive pulmonary vasoconstriction and right ventricular hypertrophy and thickening peripheral pulmonary vasculature. In the present study, rats exposed to hypoxic atmosphere showed an increase in mPAP accompanying with remodeling of vasculature. The findings are consistent with results of pervious preclinical and clinical researches supporting the statement that hypoxia induces high blood pressure and vascular remodeling response (5-8). Responding to hypoxia, a variety of cell residing in pulmonary artery such as smooth muscle cells and fibroblasts undergoes rapid expansion and contributes to vessel wall thickening (9, 10). Our histological data also support the idea that hypoxia induces inflammation and infiltration of leukocyte which contributes to the vascular thickening. We also found that serum level of TNF- $\alpha$  was elevated in response to chronic hypoxia exposure, suggesting the presence of systemic inflammation. This finding is in agreement with previous studies in which hypoxia stimulated the release of inflammatory mediators such as nitric oxide and cytokines. Moreover, we observed that the changes in pulmonary vasculature have significant influence on pulmonary blood flow and right heart function resulting in hypertension and heart failure, in consistent with previous studies.

Cardiac sympathetic nerves are involved in the regulation of cardiac performance and peripheral circulation (11, 12). It is documented that the pulmonary vasculature is innervated by the autonomic nervous system. There is accumulating evidence indicating that chronic hypoxia induce sympathetic activity leading to high vasoconstriction



**Figure 2.** Effect of TEB on CREB expression in lung tissue of hypoxia-exposed rats. Animals were exposed to either normoxia or hypoxia atmosphere for 3 weeks and treated with TEB or saline. After treatments, total protein extraction was carried out to determine CREB by immunoblotting. Lane 1: normoxia; lane 2: hypoxia; Lane 3: hypoxia/ ropivacaine and lane 4: hypoxia/ saline.  $\beta$ -actin was used as internal control. CREB: cAMP responding-element binding protein

**Table 3.** Effect of TEB on mRNA expression of CREB in lung tissue of hypoxia-exposed rats

	Normoxia	Hypoxia	Hypoxia/ ropivacaine	Hypoxia/ saline	P
CREB mRNA	$0.792 \pm 0.044$	$1.209 \pm 0.046$	$0.874 \pm 0.066$	$1.137 \pm 0.111$	$< 0.005$

All values are expressed as mean  $\pm$  SD (n = 12); CREB: cAMP responding-element binding protein

(13-17). During hypoxia, oxygen sensitive chemoreceptors in the carotid body are stimulated leading to increased efferent sympathetic outflow in humans (17, 18)). The resulting rise in vasoconstrictor drive is considered to counteract hypoxia-induced vasodilatation and maintain arterial blood pressure (19, 20). Chronic exposure to hypoxia is attributed to persistent chemoreflex activation. Unlike hypobaric hypoxia to which human body is physically acclimated, chronic hypoxia associated with lung disease contributes to PAH due to a vicious cycle of sympathoexcitation in pulmonary arteries. Cardiac sympathetic blockade with thoracic epidural anesthesia has been reported to intervene the vicious cycle of myocardial dystrophy (18-20). Ishibe et al reported that sympathetic blockage by thoracic epidural anesthesia enhanced the haemodynamic changes in dog with lobar hypoxia (24). Similar findings have been reported using different models by other researchers (25, 26). Our study, for the first time showed that TEB improves not only hemodynamic changes but also the progression of vasculature remodeling. Our data revealed that TEB significantly improved mPAP and pulmonary vascular remodeling in rats exposed to chronic hypoxic conditions. It is suggested that TEB reduces hypoxia-induced sympathetic activation, resulting in an attenuation of PAH progression. In addition, we observed that TEB ameliorated changes in serum levels of cGMP and TNF- $\alpha$ . It is indicated that TEB treatment improves hypoxia-induced pulmonary vasoconstriction and inflammation contributing to worsening of PAH.

Cyclic AMP responding-element binding protein is a hypoxia-responsive transcription factor and the expression of CREB has been considered to indicate the degree of PAH regarding SMC proliferation. CREB is required for transcriptional activation of hypoxia inducible factor (HIF) in hypoxic background. Our results showed that CREB was upregulated in response to chronic hypoxia at mRNA and protein levels. The result showing elevated expression of CREB in lung tissue of hypoxic rats was ameliorated in the presence of ropivacaine, suggesting that TEB abolishes hypoxia-induced upregulation of CREB with result of attenuated vascular remodeling.

## Conclusion

By acting on the autonomic nervous system, TEB not only attenuates blood pressure but also reverses vascular remodeling in hypoxia-induced PAH *in vivo*. In addition, TEB can affect hypoxia-associated inflammation. Collectively, we have presented *in vivo* findings strongly suggesting TEB as a beneficial therapy with great potential for treating patients with hypoxic PAH.

## Conflict of interests

No external funding and no competing interests declared.

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