Original Article

Hypoxia-inducible factor 1 alpha contributes to pulmonary vascular dysfunction in lung ischemia-reperfusion injury

Xin Zhao¹, Yanwu Jin¹, Haibo Li², Zhigang Wang³, Wanlin Zhang⁴, Chang Feng¹

¹Department of Anesthesiology, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, China; ²Operating Room, Jinan Central Hospital, Affiliated to Shandong University, 105 Jiefang Road, Jinan 250013, China; ³Department of Anesthesiology, Qilu Hospital of Shandong University, 107 Wenhua West Road, Jinan 250012, China; ⁴Department of Oral Health, Qianfoshan Hospital of Shandong Province, Affiliated to Shandong University, 16766 Jingshi Road, Jinan 250014, China

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Abstract: The revascularization therapy of pulmonary embolism is associated with ischemia-reperfusion (IR) injury. However, the effect of IR injury on pulmonary arterial endothelial function has not been elucidated. Male Sprague-Dawley rats were divided into a control, an IR and an IR plus hypoxia-inducible factor 1 alpha (HIF-1α) stabilizer DMOG group. We found that the acetylcholine (ACh)-induced relaxation was dramatically reduced in pulmonary arteries from IR-injured rats compared with controls (P < 0.01). Interestingly, pre-treatment with the DMOG significantly improved ACh-stimulated pulmonary arterial dilatation (P < 0.01). The protein expression of HIF-1α in pulmonary artery was significantly down-regulated by IR injury (P < 0.01). Moreover, DMOG remarkably reversed IR-induced down-regulation of HIF-1α (P < 0.01). There was no difference in ACh-stimulated relaxation of endothelium-denuded or L-NMMA-treated pulmonary arteries among the three groups. The bioavailability of nitric oxide (NO) and the phosphorylation level of inducible NO synthase (iNOS) in pulmonary artery were significantly decreased by IR injury (both P < 0.01), which were reversed by DMOG (P < 0.05 or P < 0.01). In addition, the levels of superoxide in pulmonary artery were not affected by the IR injury as well as IR injury plus administration with DMOG. The present study demonstrated that HIF-1α contributes to pulmonary vascular dysfunction in lung IR injury.

Keywords: Hypoxia-inducible factor 1 alpha, pulmonary vascular dysfunction, lung, ischemia-reperfusion injury, inducible nitric oxide synthase

Introduction

Chronic thromboembolic pulmonary hypertension (CTPH) accounts for substantial morbidity and mortality [1]. The pathogenesis of the CTPH has not yet been fully elucidated [2]. Acute pulmonary thromboembolism secondary to deep venous thrombosis may serve as the inciting event that increases pulmonary vascular resistance resulting in pulmonary hypertension [3]. However, the observation that some patients who receive reperfusion therapy also suffer the CTPH [4], suggesting that there are some factors associated with reperfusion that are important in the development of the CTPH.

Pulmonary arterial dysfunction leads to increased vascular resistance and pulmonary hypertension [5]. The revascularization therapy of pulmonary artery might be associated with endothelial ischemia-reperfusion (IR) injury, leading to vascular dysfunction and subsequently pulmonary hypertension. The previous study demonstrated that forearm occlusion and recovery induced conduit artery endothelial dysfunction, as assessed by brachial artery flow-mediated dilation [6]. However, the effect of IR injury on pulmonary arterial endothelium has not been elucidated.

The mechanisms underlying IR injury are incompletely understood, but increases in oxidative stress and decreases in the bioavailability of nitric oxide (NO) play crucial roles [7]. Importantly, pulmonary hypertension is partially
attributed to the decrease in NO and supplement of NO has been viewed as a potent therapeutic strategy [8, 9]. However, the exact role of NO in pulmonary endothelial IR injury has not been understood. The inducible NO synthase (iNOS) is an important producer of NO that can be quenched by superoxide. Additionally, hypoxia-inducible factor 1 alpha (HIF-1α) has been associated with the IR injury and the activation of iNOS.

The present study was designed to investigate the effect of IR injury on pulmonary arterial endothelium and the underlying mechanisms.

Materials and methods

Animals

The experiments conform to the Guide for the Care and Use of Laboratory Animals (American National Institutes of Health Publication No. 85-23, revised 1996), and all the procedures were approved by the Institutional Ethics Committee. Male Sprague-Dawley rats (150-200 g) were purchased from the local animal center and randomly divided into a control, an IR and an IR plus HIF-1α stabilizer DMOG group. All the rats were housed under a 12 h/12 h day/night cycle, with ad libitum food and water.

Lung I/R injury

Rats were randomly divided into a control, an IR and an IR plus DMOG group. Animals in IR plus DMOG group were given an intraperitoneal injection of DMOG (40 mg/kg in 1 mL saline, n = 10) 24 hours before procedure. Rats in IR group were given an intraperitoneal injection of saline (1 mL, n = 10) 24 hours before procedure. Animals in IR and IR plus DMOG groups were underwent IR procedure while the rats in control group were underwent sham surgery. The lung IR injury was induced though 60 min ischemia by ligation of the pulmonary artery and 24 hours reperfusion [10]. Rats were initially anaesthetized with pentobarbital given intraperitoneal (30 mg/kg) and maintained with pentobarbital given a continuous intravenously infusion of 0.1 mg/kg/min. After endotracheal intubation, the mechanical ventilation (using MMS RET 107 ventilator; MMS, Pau, France) was performed with tidal volume of 10 mL/kg, 70 breaths/min and supplementation with 100% oxygen. A midline thoracotomy was performed under sterile conditions, the pericardium was removed, and the left pulmonary artery was ligated using an 8-0 prolene suture. A small piece of polyethylene tubing was used to secure the ligation without damaging the artery. After 60 min of ischemia, the left pulmonary artery occlusion was released and reperfusion occurred. The chest was closed and the ventilator removed to restore normal respiration. Sham-operated animals were submitted to the same surgical protocol as described but without ligation.

Vascular reactivity

Vascular reactivity of freshly isolated pulmonary artery was studied in myograph (Danish Myo Technology, Denmark) as described [11]. The pulmonary arteries were dissected out and cleaned of connective tissue. If needed, the endothelium of some rings was removed by gently rubbing the lumen with human hair. Pulmonary artery rings (2.5 mm in length) were mounted in myograph. Each ring was bathed in Krebs solution containing (mmol/L): 118 NaCl, 25 NaHCO₃, 5.4 Glucose, 4.7 KCl, 2.5 CaCl₂, 1.2 KH₂PO₄ and 1.2 MgSO₄, aerated with 95% O₂ and 5% CO₂ at 37°C (pH 7.4). The tension was set to the estimated in vivo internal circumference. After 60 min stabilization, functional integrity of segments was confirmed by contraction to KCl (60 mmol/L). The function of endothelium was confirmed by a relaxant response to acetylcholine (ACh, 1 µmol/L) in segments pre-contracted with phenylephrine (1 µmol/L). Rings relaxing over than 80% were defined as endothelium-intact, while those relaxing less than 10% were considered as endothelium-denuded. The endothelium-dependent and endothelium-independent function was estimated by the analysis of the relaxant response to cumulative addition of acetylcholine and nitroglycerin (NTG) (10⁻⁹-10⁻⁴ mol/L) after pre-contraction with 10⁻⁷ mol/L phenylephrine. Some experiments were carried out in the presence of the iNOS inhibitor L-NMMA.

Western blotting

The protein expressions of HIF-1α, total iNOS, phosphorylated iNOS (p-iNOS) and β-actin were detected by Western blotting [12]. Pulmonary arterial tissues were homogenized in high-salt buffer containing NaCl 600 mmol/L, 3-(N-
mopholino) propanesulfonic acid 40 mmol/L, dithiothreitol 1 mmol/L, leupeptin 1 µg/mL, aprotinin 1 µg/mL, phenylmethylsulfonfyl fluoride 50 mmol/L. The total protein concentrations were examined with Bio-Rad protein assay. Proteins were separated using 10% sodium dodecyl sulfate polyacrylamide gel and transferred to nitrocellulose membranes (EMD Millipore Corporation, Billerica, MA, USA) at 100 V for 60 min. Membranes were blocked with 5% non-fat dry milk for 8 hours at 4°C. Membranes were incubated with primary rabbit anti-HIF-1α, anti-iNOS, anti-p-iNOS and anti-β-actin antibodies (diluted 1:1000, Santa Cruz Biotechnology, USA) overnight at 4°C. Membranes were then incubated with secondary goat anti-rabbit horseradish peroxidase antibodies (diluted 1:2000, Santa Cruz Biotechnology, USA) for 60 min at room temperature and the bound antibody was visualized using a colored reaction. The relative band densities were quantified by densitometry using the Multi-Analyst software package (Bio-Rad). Equal loading of protein was confirmed by measuring β-actin or total iNOS expressions. Each sample was processed three to six times.

**NO bioavailability**

The NO bioavailability in freshly isolated pulmonary artery were assessed using 4,5-diamino-fluorescein (DAF-2 DA, Sigma-Aldrich, USA) as described previously [13]. Pulmonary arteries were gently harvested and the surrounding connective tissue was removed in ice-cold gassed (95% O₂ and 5% CO₂) Krebs solution under an anatomical microscope as previously described. The arteries were opened longitudinally and placed on a cover slip. Pulmonary segments were loaded with 5 µmol/L DAF-2DA for 45 min at 37°C in Krebs solution. The arteries were then rinsed three times with fresh Krebs solution and were placed under a fluorescence microscope (Nikon TE2000, Nikon Corporation, Japan) outfitted with a fluorescein isothiocyanate filter set. Images were acquired and the fluorescence intensity was analyzed using the software NIS-Elements 3.0 (Nikon Corporation, Japan).

**Superoxide level**

Pulmonary arteries were prepared in the manner described above for the DAF-2 assay, and
the arteries were loaded with dihydroethidium (DHE, Sigma-Aldrich, USA) diluted in Krebs (40 μmol/L) for 30 min at 37°C followed by a 15 min wash in Krebs solution [14]. The arteries were then placed under a fluorescence microscope (Nikon TE2000, Nikon Corporation, Japan) outfitted with a rhodamine filter set. Images were acquired and the fluorescence intensity was analyzed using the software NIS-Elements 3.0 (Nikon Corporation, Japan).

Statistical analysis

Data are presented as means ± SEM. Comparisons between groups were determined by one-way ANOVA with Student’s t-test post hoc test (SPSS Inc., Chicago, IL). Results were considered significant when p value was less than 0.05. Two-sided P values below 0.05 were considered as statistically significant.

Results

Role of HIF-1α in IR-induced endothelial dysfunction of pulmonary artery

This acetylcholine-induced relaxant response was dramatically reduced in pulmonary arteries from IR-injured rats compared with controls (P < 0.01) (Figure 1A). Interestingly, pre-treatment with the HIF-1α stabilizer DMOG significantly improved acetylcholine stimulated pulmonary arterial dilatation (P < 0.01) (Figure 1A). In addition, nitroglycerin-induced vascular relaxations were not affected by the IR injury as well as IR injury plus administration with DMOG (Figure 1B). The protein expression of HIF-1α in pulmonary artery was significantly down-regulated by IR injury (P < 0.01). Moreover, DMOG remarkably reversed IR-induced down-regulation of HIF-1α (P < 0.01) (Figure 1C).

Endothelium dependence

The relaxant response induced by acetylcholine was partially inhibited by endothelial denudation in these three groups. Moreover, there was no difference in acetylcholine stimulated relaxation of endothelium-denuded pulmonary artery among the three groups (Figure 2A). Additionally, endothelial denudation did not affect nitroglycerin-induced vascular relaxation (Figure 2B).

Role of iNOS

The relaxation induced by acetylcholine was significantly inhibited by iNOS inhibitor L-NMMA in these three groups (Figure 3A). In addition, nitroglycerin-induced vascular relaxations were not affected by L-NMMA in these three groups (Figure 3B). The phosphorylation level of iNOS in pulmonary artery was significantly down-regulated by IR injury (P < 0.01). Moreover, DMOG remarkably reversed IR-induced down-regulation of phosphorylated iNOS (P < 0.01) (Figure 3C).

Role of NO and superoxide

The bioavailability of NO in pulmonary artery was significantly decreased by IR injury (P < 0.01). However, IR-induced decrease in NO level was obviously attenuated by DMOG (P < 0.05) (Figure 4A and 4B). In addition, the levels
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Discussion

There are two novel findings in the present study. Firstly, endothelium-dependent pulmonary arterial dilatation but not endothelium-independent relaxation was significantly impaired by the lung IR injury accompanied by the down-regulation of HIF-1α, de-phosphorylation of iNOS, and reduction in NO bioavailability. Secondly, pre-treatment with HIF-1α stabilizer DMOG remarkably attenuated IR-induced endothelial dysfunction accompanied by restoring the expression of HIF-1α, phosphorylation of iNOS, and increase in NO bioavailability.

CTEPH is the result of single or recurrent pulmonary embolism [15, 16]. Revascularization strategies including fibrinolysis, catheter-assisted embolectomy and surgical embolectomy significantly improved the treatment of acute massive and submassive pulmonary embolism [17-19]. However, these therapeutic methods can't prevent the incidence of pulmonary hypertension [20]. The underlying mechanism responsible for post-embolism pulmonary hypertension has not been elucidated. Vascular dysfunction plays an important role in the development of pulmonary hypertension [21]. Previous study has demonstrated that IR injury significantly increased both pulmonary vascular resistance and vascular permeability in isolated lung [22]. However, the endothelial function of pulmonary artery after lung IR injury has not been investigated. The present study demonstrated that lung IR injury didn't affect nitroglycerin-induced endothelium-independent vascular dilatation but caused a significant impairment of acetylcholine-induced vascular relaxation which was abolished by endothelial denudation or incubation with the iNOS inhibitor L-NMMA, indicating that IR-induced vascular dysfunction might be endothelium dependent and due to reduction in NO bioavailability. These findings suggest that pulmonary arterial occlusion-reperfusion injury might induce en-
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dotheilial dysfunction and partially contribute to the development of pulmonary hypertension.

HIF-1, a transcription factor composed of oxygen-labile HIF-1α and constitutive HIF-1β subunit, is responsible for the response to hypoxia or ischemia. The activation of HIF-1α plays a protective role in IR injury of several tissues through regulating the inflammation [23]. The present study has demonstrated that down-regulation of HIF-1α might contribute to the development of IR injury of pulmonary artery. Moreover, HIF-1α stabilizer DMOG might become a potent agent against IR injury by restoring the expression of HIF-1α.

The NO bioavailability has been viewed as an important factor in the regulation of IR injury. The NO level is dependent on the production by NOS and the elimination through quenching by superoxide [24]. The activation of iNOS is linked to the expression of HIF-1α [25]. The present study has demonstrated that the down-regulation of HIF-1α was associated to the de-phosphorylation of iNOS. Additionally, the restore of HIF-1α expression induced by DMOG was linked to the phosphorylation of iNOS. Moreover, the phosphorylation level of iNOS was associated to the level of NO in pulmonary arterial endothelium. However, the level of superoxide was not affected by IR injury or DMOG treatment. These findings suggest that the regulatory effect of HIF-1α on the pulmonary endothelial function in lung IR injured rats might be attributed to the iNOS/NO pathway.

In conclusion, the present study demonstrated that HIF-1α contributes to pulmonary vascular dysfunction in lung IR injury and HIF-1α stabilizer DMOG can attenuate IR-induced endothelial function through iNOS/NO pathway.

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Figure 4. Role of nitric oxide (NO) and superoxide. A: NO bioavailability detected by DAF-2 DA staining in pulmonary arteries of rats from a control, an ischemia-reperfusion (IR) and an IR plus HIF-1α stabilizer DMOG group. B: Summarized data showing the average fluorescence intensity in pulmonary arteries from each group. C: Superoxide anion was detected by DHE staining in pulmonary arteries of rats from a control, an IR and an IR plus DMOG group. D: Summarized data showing the average fluorescence intensity in pulmonary arteries from each group. *P < 0.05, **P < 0.01.
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Disclosure of conflict of interest

None.

Address correspondence to: Dr. Xin Zhao, Department of Anesthesiology, The Second Hospital of Shandong University, 247 Beiyuan Road, Jinan 250033, China. E-mail: dr_xinzhao@yeah.net

References


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