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Suppression of plasminogen activator inhibitor-1 by inhaled nitric oxide attenuates the adverse effects of hyperoxia in a rat model of acute lung injury

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ABSTRACT

Introduction: Locally increased expression of plasminogen activator inhibitor-1 (PAI-1) in acute lung injury (ALI) is largely responsible for fibrin deposition in the alveolae and lung microvasculature. In vitro, nitric oxide (NO) effectively suppresses the ischemic induction of PAI-1. We aimed to investigate the effects of inhaled NO on PAI-1 expression in ALI in a rat model with and without hyperoxia.
Materials and Methods: Healthy adult rats were primed with lipopolysaccharide (LPS) via an intraperitoneal challenge followed by a second dose of LPS given intratracheally to induce ALI (LPS group), whereas the control groups were given sterile saline. All groups were allocated to subgroups according to gas exposure: NO (20 parts per million, NO), 95% oxygen (O), both (ONO), or room air (A). At 4 h, 24 h, 48 h (after 4 h or 24 h exposure to the various gases, 24 h gas intervention and then observation until 48 h), the rat lungs were processed and PAI-1 protein and mRNA expression, histopathological lung injury scores and fibrin deposition were evaluated.
Results: At 4 and 24 h, inhaled NO caused the PAI-1 mRNA levels in the LPS-NO and LPS-ONO subgroups to decrease compared with the untreated LPS subgroups. At 48 h, higher PAI-1 mRNA levels than those of the corresponding control subgroup were only observed in the LPS-O subgroup, and these values were lower in the LPS-ONO subgroup than in the LPS-O subgroup. The trends of the PAI-1 protein levels mirrored those of PAI-1 mRNA. At 48 h, PAI-1 protein levels in the LPS-NO and LPS-ONO subgroups were decreased compared with those in the untreated LPS subgroups. The histopathological lung injury scores and fibrin deposition in LPS subgroups that inhaled NO showed a decreasing trend compared with the untreated LPS subgroups.
Conclusions: Inhaled NO can suppress elevated PAI-1 expression in rats with ALI induced by endotoxin. Although exposure to high-concentration oxygen prolongs the duration of PAI-1 mRNA overexpression in ALI, inhaled NO can reduce this effect and alleviate both fibrin deposition and lung injury.

Introduction

Acute lung injury (ALI) and acute respiratory distress syndrome (ARDS) are common life-threatening causes of acute hypoxemic respiratory failure that are associated with both intrapulmonary and extrapulmonary disorders in the absence of left atrial hypertension [1,2]. ALI/ARDS is a major cause of morbidity and mortality among critically ill adults and children. In adult patients with ALI/ARDS, mechanical ventilation with a lower tidal volume has been proven to reduce mortality [3], and a multicenter clinical trial using an effective lung protection strategy in pediatric ALI/ARDS is warranted [4,5]. Insight into the pathophysiology of ALI can help to identify risk factors and develop new therapeutic interventions.

It is well established that ALI/ARDS is characterized by local and intense inflammatory responses. Fibrin deposition in the alveolar and lung microvasculature likely results from disordered coagulation and fibrinolysis, which are triggered by inflammation in ALI/ARDS [6]. Impaired coagulation and fibrinolysis may be an important pathogenetic and prognostic determinant of mortality in ALI/ARDS [7]. Locally increased amplification of plasminogen activator inhibitor-1 (PAI-1) is largely responsible for the fibrin deposition in ALI.

Clinical trial data indicate that higher plasma PAI-1 levels are associated with increased mortality and fewer ventilator-free days among adults and pediatric patients with ALI/ARDS [8,9]. A single guanosine insertion/deletion (4G/5G) polymorphism in the promoter region of the PAI-1 gene may play an important role in the regulation of PAI-1 expression. ALI/ARDS patients homozygous for the 4G allele of the PAI-1 gene experience increased 28-day mortality [10].

Accordingly, PAI-1 inhibitors may be used as a new therapeutic intervention for ALI. The lung epithelium appears to be an important

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site for fibrin deposition in alveolae [11]. Therefore, targeting the lung epithelium with inhaled therapies to modulate the intra-alveolar expression of PAI-1 warrants further study. Inhaled nitric oxide (iNO) has been demonstrated to improve oxygenation via selective pulmonary vasodilation to modulate ventilation-perfusion mismatching in refractory ARDS [12]. In adult ARDS, inhaled NO has been unable to significantly improve outcomes [13]. However, recent studies in pediatric ARDS have demonstrated that the use of inhaled NO results in a significant decrease in the duration of ventilation and a significant increase in extracorporeal membrane oxygenation (ECMO)-free survival [14]. The mechanism responsible for these improved outcomes in ARDS with inhaled NO may involve the role of nitric oxide in modulating coagulation and inflammation. In vitro, NO effectively suppresses the ischemic induction of PAI-1 [15]. In the present study, we aimed to characterize the effects of early intervention with inhaled nitric oxide on the expression of PAI-1 in a rat model of ALI induced by lipopolysaccharide (LPS).

Inhaled NO is usually used in combination with high-concentration oxygen in critical ARDS. An animal study showed that the lungs of mice exposed to hyperoxia overproduce PAI-1 [16]. We additionally examined changes in PAI-1 expression in ALI in response to inhaled NO together with high-concentration oxygen.

Materials and Methods

Animals

All studies were performed on specific pathogen-free male Sprague-Dawley rats (Super-B&K Laboratory Animal Corp., Ltd., Shanghai, China) at 180–220 g body weight. The experimental protocol was approved by the Ethics Committee of the Children's Hospital of Fudan University and complied with the Chinese national regulations on experimental animal care. Animals were anesthetized by an intraperitoneal administration of 5–10 mg ketamine hydrochloride (Hengrui Medicine Corp., Ltd., Jiangsu, China) per 100 g body weight in 200 μL.

A total of 186 healthy adult rats were randomly allocated to a lipopolysaccharide (LPS) group or a normal control group (C). The LPS group was primed with LPS (E. coli O111B4, Sigma-Aldrich Co., USA) 0.1 mg/kg via intraperitoneal challenge followed by a second dose of LPS 1 mg/kg instilled intratracheally as previously described [17,18] 16 h later to induce ALI. The C group received a sterile saline injection in an identical manner. Both the LPS and C groups were then randomly assigned to subgroups according to exposure: room air (Air), 95% oxygen (O), room air and 20 ppm NO (NO), or 95% oxygen and 20 ppm NO (ONO). NO gas was prepared at 1,000 ppm and provided with a mass flow controller. The NO concentration was measured with an NO/NO2 electrochemical analyzer (NOxBOX Plus, Bedfont Scientific, Inc., Rochester, UK). At the 4 h, 24 h, and 48 h time points (after 4 h and 24 h exposure to the different gases, and the 24 h gas intervention was followed by observation until 48 h), the rats were sacrificed (n = 6–8 in each C subgroup at each time point; n = 8–10 in each LPS subgroup at each time point), and their lungs were processed.

Quantitative Real-Time Reverse Transcriptase-Polymerase Chain Reaction

The right lower lung lobe was homogenized in TRIzol (Invitrogen, Carlsbad, CA, USA) to extract the total RNA, which was then reverse transcribed into complementary DNA (cDNA). Rat PAI-1 mRNA was determined with a forward primer 5' - TCTCCGCCATCACCAACATT - 3' and a reverse primer 5' - GAGAGAACTTAGGCAGGATGAGG - 3'. β-Actin was used as the reference gene with the forward primer 5' - AACCCTAAGG CCAACAGTGAAAAG - 3' and reverse primer 5' - TCATGAGGTAGTCTGT GAGGT - 3'. The relative quantities of mRNA are presented as (the common logarithm of the copy number of the targeted gene $\times 10^7$)/(copy number of β-actin).

Immunohistochemistry

Specimens of the left lung lobe were fixed with paraformaldehyde and then embedded in paraffin. Sections were cut at 5 μm, collected on aminopropyltriethoxysilane-coated slides, and dried for 6 to 24 hours at 45 °C. After deparaffinization and rehydration, the slides were incubated with rabbit polyclonal antibodies against PAI-1 (1:50, Boster, China). Antigens were visualized using the SABC Staining System (Boster, China) according to the manufacturer's instructions. Negative controls were stained by substitution of the primary antibody with non-immune immunoglobulin. Positive staining was recognized under a microscope by the brown color of diaminobenzidine.

A Leica DMRA2 microscope (Leica, Germany) was used to acquire images of the sections at × 400 magnification. Five randomly selected fields per section were analyzed. Images were processed using the Image-Pro Plus 6.0 software (Media Cybernetics, Inc., Silver Spring, MD, USA) and masked for any empty areas and air spaces. The integral optical density (IOD) value of positive PAI-1 protein in the cytoplasm of rat lungs and the area of lung tissue (Area) were analyzed with the same image analysis software as previously described [19]. The relative quantity of PAI-1 protein is presented as the common logarithm of the mean density (IOD/Area) of the targeted protein ($\times 10^3$).

Lung Histopathology and Morphometry

The lungs from each subgroup were removed and fixed for another 12 h in paraformaldehyde buffer at 4 °C. Representative lung tissue blocks from all lung lobes were embedded in paraffin. Sections of 5-μm thickness stained with hematoxylin and eosin were examined under light microscopy for lung histopathological abnormalities and scored for inflammation, hemorrhage, edema, atelectasis, overinflation, microthrombosis and small airway injury. A score scaled from 0 to 4 represents the severity: 0 for no or very minor injury; 1 for injury in

Table 1
The body weight and age of the rats.

Subgruop	Body Weight (g)			Age (d)		
	4 h	24 h	48 h	4 h	24 h	48 h
C-A	199.0 ± 15.2	191.3 ± 13.1	204.4 ± 18.3	40.2 ± 2.0	39.8 ± 2.2	41.0 ± 1.7
LPS-A	214.0 ± 11.0	198.8 ± 8.5	203.6 ± 7.02	41.4 ± 2.1	40.6 ± 2.1	40.8 ± 1.8
C-NO	197.7 ± 16.3	195.8 ± 14.1	194.8 ± 15.0	40.5 ± 1.0	40.8 ± 1.7	39.6 ± 1.5
LPS-NO	200.0 ± 13.3	199.2 ± 14.8	200.0 ± 15.3	40.8 ± 1.6	40.7 ± 2.2	40.2 ± 1.5
C-O	197.6 ± 12.9	208.3 ± 10.7	201.0 ± 15.6	40.2 ± 1.9	41.4 ± 1.8	40.2 ± 2.2
LPS-O	205.4 ± 21.6	202.2 ± 14.1	201.6 ± 13.5	40.8 ± 1.8	41.0 ± 1.0	40.8 ± 1.9
C-ONO	193.6 ± 10.3	197.2 ± 14.4	196.2 ± 14.3	40.0 ± 2.0	40.7 ± 2.7	40.0 ± 2.1
LPS-ONO	195.2 ± 15.7	198.0 ± 16.5	195.6 ± 12.2	40.6 ± 1.7	40.0 ± 1.9	39.8 ± 2.0

Subgroup definitions: C-A, control exposed to air; C-NO, control with 20 ppm NO; C-O, control with 95% oxygen; C-ONO, control with 95% oxygen and 20 ppm NO; LPS-A, LPS treatment with air; LPS-NO, LPS with 20 ppm NO; LPS-O, LPS with 95% oxygen; LPS-ONO, LPS with 95% oxygen and 20 ppm NO. Values are means ± SD in each subgroup. At the 4 h, 24 h, and 48 h time points (after 4 h and 24 h of exposure to the various gases, 24 h gas intervention and then observation until 48 h), the rats were sacrificed (n = 6–8 in each C subgroup at each time point; n = 8–10 in each LPS subgroup at each time point).