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Inhaled Nitric Oxide Attenuates Endotoxin Induced Acute Respiratory Distress Syndrome

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Abstract

Acute respiratory distress syndrome (ARDS) is characterized by acute inflammation with the disruption of the respiratory epithelial barrier representing a major emergency in patients requesting acute therapeutic intervention. Here we asked whether nitric oxide (NO) gas may attenuate ARDS. Using a portable NO generator we report that NO inhalation attenuated endotoxin (LPS) induced ARDS in mice. We find that inhalation of NO reduced pro-inflammatory cytokines such as IL-1b, IL-6, as well as myeloperoxidase levels and neutrophil recruitment in the airways. Consequently, respiratory barrier injury was attenuated with diminished protein leak and morphological changes in the lung. Therefore, the data suggest that early inhalation of NO may be an interesting therapeutic approach to be considered in patients with ARDS.

Keywords: ARDS, endotoxin, nitric oxide, NO portable generator

Introduction

Acute respiratory distress syndrome (ARDS) is a severe respiratory failure with epithelial injury and pneumonia which can be caused by many pathogens such as bacteria and virus including the severe acute respiratory corona virus (SARS-CoV) [1]. A common feature in ARDS patients is lung hyper-inflammation with an excessive release of inflammatory mediators including cytokines which play an important role in the evolution of the disease. The optimal management of ARDS patients is complex and experts evaluate frequently efficacy and safety of therapeutic strategies [2].

The lung administration of endotoxins from Gram-negative bacteria induces an acute respiratory distress syndrome with disruption of the epithelial barrier, neutrophil infiltration and accumulation of inflammatory enriched-mediators[3]. The LPS lung instillation model can be used as a model system for the analysis of the physiopathology of ARDS as well as its mitigation.

Nitric oxide (NO) is known to be an anti-inflammatory molecule with many other functions such as anti-microbial and regulation of the pulmonary vascular function [4-6]. In order to test if

NO plays a role in experimental ARDS, we have treated mice with inhaled NO gas from a portable generator and observed that at early time after instillation of LPS, NO attenuates LPS induced ARDS.

Methods

Mice

C57BL/6 wild-type mice from pathogen-free animal facility at the Centre National de la Recherche Scientifique were used. For experiments, adult 8-10 weeks old mice were kept in isolated ventilated cages. All animal experimental protocols complied with French ethical and animal experiments regulations (see Charte Nationale, Code Rural R 214-122, 214-124 and European Union Directive 86/609/EEC) and were approved by the "Ethics Committee for Animal Experimentation of CNRS Campus Orleans" (CCO), registered (N°3) by the French National Committee of Ethical Reflexion for Animal Experimentation (CLE CCO 2013-1006).

Model of ARDS and NO from TAS Plus exposure

Endotoxin/LPS (*Escherichia coli*, serotype O111:B4, Sigma-Aldrich) was administered intranasally at a dose of 1µg per mouse in 40µl under light isoflurane anesthesia to groups of 5 mice, and the study was done twice. Mice were analyzed at 6h. Broncho-alveolar lavage (BAL) with total and differential cell counts, protein, cytokine and histology was performed on the lung. Saline was administered intranasally to control groups and analyzed 6h after.

NO was generated by the INJECT+MATIC TAS Plus generator (www.injectmatic.com) already reported [7, 8]. Within the 10 minutes after LPS or saline administration, mice were placed in a transparent plastic chamber or left in the ambient air. In the following 5 minutes, the air inside the plastic chamber contained 20 ppm of NO as monitored by chemiluminescence using an Eco Physics CLD 700 AL analyzer. NO_2 was always below 0.3 ppm. Animals were exposed during 6 h and then sacrificed for analysis.

Quantification of protein, Evans Blue (EB) and cellular infiltration in BAL

BAL was performed by four lavages of lung with 500 µL of saline solution via a cannula introduced into mice trachea. BAL fluids were centrifuged at 400× *g* for 10min at 4°C, the supernatants were stored at –20°C for analysis, and pellets were recovered to prepare cytospin (Thermo Scientific, Waltham, MA, USA) on glass slides followed by a Diff-Quik (Merz& Dade A.G., Dudingen, Switzerland) staining. Differential cell counts were performed with at least 300 cells.

Vascular leakage was quantified by protein and Evans Blue concentration in the BAL fluid. EB in BAL was measured 45 min after intravenous injection of 0.3% of EB, by absorbance at 460 nm as described [9]. Extravasation is expressed as micrograms of EB per millilitre volume of BAL supernatant.

Microscopy

The left lobe of lung was fixed in 4% buffered formaldehyde and paraffin embedded under standard conditions. Tissue sections (3 µm) were stained with standard H&E and PAS. Histological score of pathology was determined by a semi-quantitative assessment from 0 to

5 for cell infiltration (with increasing extent). The slides were blindly examined by two investigators with a Leica microscope (Leica, Germany).

Enzyme-linked ImmunoSorbant Assay (ELISA)

BAL and homogenized lungs were tested for IL-1b, IL-6, TNF and CXCL1 using commercial ELISA kits (eBiosciences, San Diego, CA, USA) according to the manufacturer's instructions.

Statistical Analysis

Data were analysed using Prism version 5 or 6 (Graphpad Software, San Diego, CA, USA). Either Mann-Whitney t tests or the parametric one-way ANOVA test with Bonferroni's multiple-comparison was used to assess significance. Values are expressed as mean ± SD.

Results

Exposure to NO generated by TAS Plus

A NO generator "INJECT+MATIC TAS Plus" has been recently described to be used for the treatment of neonatal hypertension[7, 8]. This NO generator is an electro-pneumatic device enriching atmospheric air in air containing NO without limitation of gas production. Pre-clinical studies carried on three different piglet models of hypertension have demonstrated its safety and therapeutic efficacy. Clinical trials have shown that NO at 20 ppm can be used to treat neonatal hypertension. The amount of NO delivered by the TAS Plus device can be easily regulated and monitored as previously described[7]. Considering the broad range of activities of NO, we asked if inhaled NO can attenuate ARDS in mice. Here, we exposed mice to NO at concentration of 20 ppm in a Plexiglas chamber during 6 h and compared to those maintained in the air ambient following LPS or saline administration.

Endotoxin induced ARDS

LPS at 1 µg by intranasal route induces acute inflammation with increased neutrophils in BALF at 6h, with a slight increase of lymphocytes, while macrophages and total cells are not different to saline control (Figure 1A).

Figure 1 LPS induced inflammatory cell recruitment and affected the and integrity of respiratory barrier.

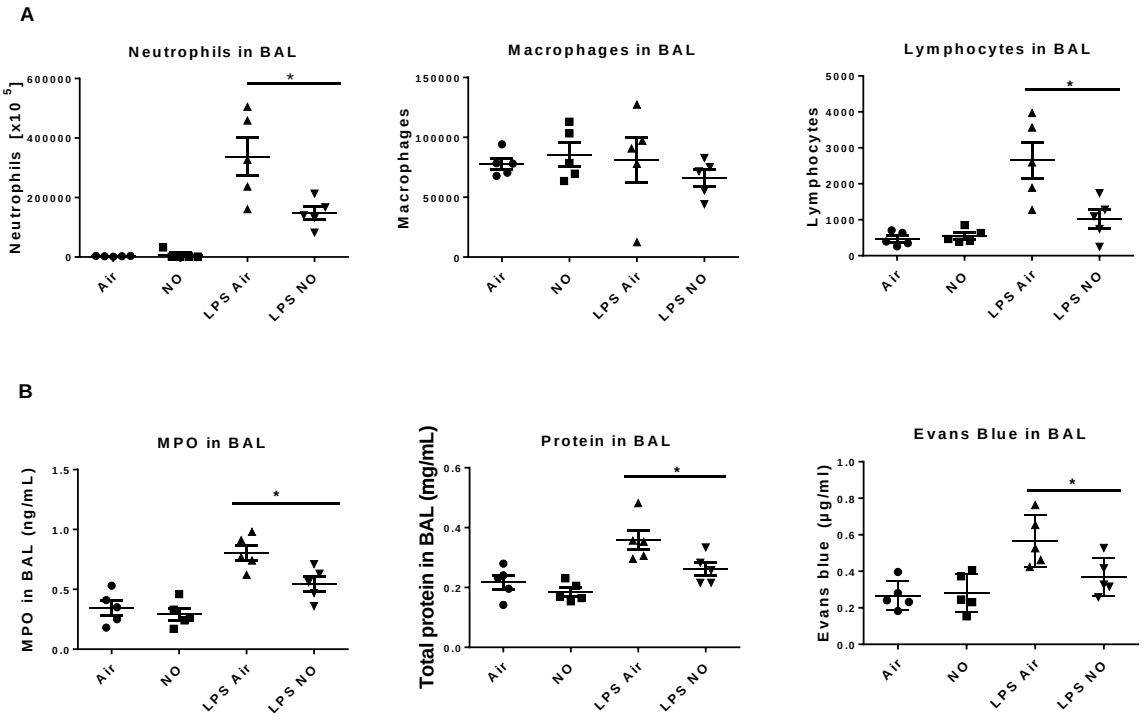
A. Neutrophils, macrophages and lymphocytes were counted in BALF
B. MPO, protein and Evans blue were evaluated in BALF

Groups of 5 female mice were intranasally administered LPS (1µg/ml) and immediately exposed to NO at 20ppm and BALF cells and fluid were analyzed at 6h. Data are representative of two independent experiments and expressed as individual spots and SD +/1SD.*$p<0.05$

The neutrophilic inflammation is confirmed by increased myeloperoxidase levels. Furthermore, increased protein levels and Evans blue leak in BALF after intravenous injection demonstrate disruption of the airway epithelium (Figure 1B).

Having established the early injury of the lung with respiratory barrier damage and inflammation we tested the potential effect of NO gas exposure by the INJECT+MATIC TAS Plus portable device.

Figure 1



NO inhalation attenuates endotoxin induced ARDS

After the LPS challenge, mice were exposed immediately to continuous NO at 20ppm in a chamber during 6h. We find reduced recruitment of neutrophils and lymphocytes, while macrophages are not different to saline control (Figure 1A). Furthermore, reduced MPO, protein levels and EB in BALF demonstrate a significant protection from airway epithelium damage (Figure 1B). Inflammatory mediators such IL-1b, IL-6 and TNF, which are increased upon LPS are significantly reduced upon NO exposure (Figure 2A).

Microscopic investigations reveal that epithelial damage and neutrophilic inflammation are drastically attenuated by NO exposure (Figure 2B).

Therefore, NO has a protective effect on acute LPS induced lung injury and inflammation.

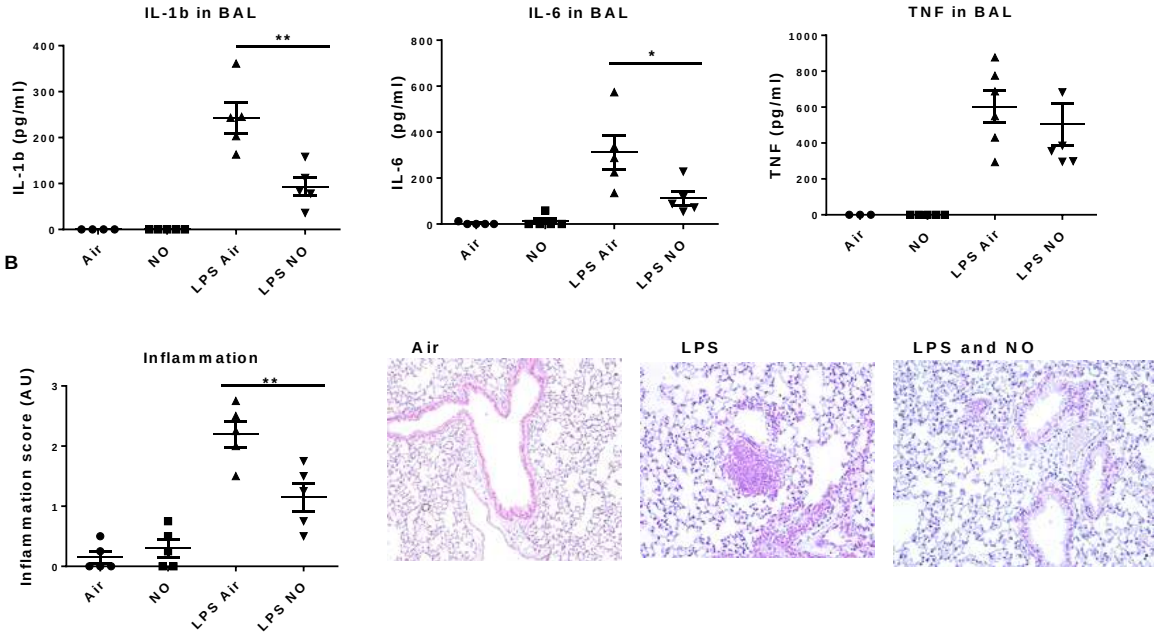
Figure 2 NO attenuated inflammatory mediators and lung inflammation

A. IL-1b, IL-6 and TNF were measured in BALF
B. Microscopy of lung and inflammatory score

Groups of 5 female mice were intranasally administered LPS (1µg/ml) and immediately exposed to NO at 20ppm and BALF cells and fluid were analyzed at 6h. Data are representative of two independent experiments and expressed as individual spots and SD +/1SD.*p<0.05, **p<0.01.

Figure 2

A



Discussion

NO, acting as a potent selective pulmonary vasodilator, is the gold standard therapy for pulmonary hypertension improving oxygenation in newborns suffering from hypoxemic respiratory failure[10]. However, the high cost of inhaled NO in gas cylinder and the complexity of the system for delivering appropriate dosage of NO are important limitations for applying this therapy in hospital centres of developing countries. In this context, we have developed a portable system named TAS Plus NO generator that provides a continuous flow of gas. This is a low cost device that has been tested in a three phases study involving evaluation of the safety, efficacy and a clinical study in human neonates suffering from pulmonary hypertension [7, 8]. Three models of pulmonary hypertension in piglets demonstrated that the new NO generator was efficient in rapidly decreasing pulmonary hypertension. Newborn babies suffering from primary and secondary pulmonary hypertension treated with the new device showed an increase in oxygenation saturation and partial pressure of oxygen. This new NO generator represents a breakthrough NO therapy for patients that do not have access to this therapy.

ARDS is a severe disease developed by patients suffering from different pathologies including sepsis and pneumonia [11]. ARDS patients present respiratory failure associated with diffuse pulmonary infiltrates and hypoxemia despite oxygen supply. The optimal management of these patients is difficult and the mortality rate is up to 40-50% [11, 12].

The present study was undertaken to explore whether inhaled nitric oxide (NO) gas attenuates LPS-induce ARDS characterized by an important recruitment of neutrophils to the lung affecting the lung function and the release of numerous inflammatory cytokines resulting in pulmonary inflammation. Our results show that NO inhalation does not affect any cytokine

expression and cellularity in the BALF of control mice. However, after LPS-induced ARDS there was a decrease of neutrophils and lymphocytes in the BALF of mice breathing 20 ppm NO compared to those maintained in the ambient air. Furthermore, MPO, protein content and vascular leak were attenuated in mice with NO. In addition, IL-1b and IL-6 levels were reduced in mouse BALF exposed to NO, however TNF just showed a small difference as this cytokine is rapidly released after LPS and 6 h does not correspond to the higher amounts of TNF in BALF [3]. Consequently, the attenuated response of neutrophils and mediators in the NO exposed animals reduced inflammatory cell infiltration in the lung.

Although the licensed indication of inhaled NO is pulmonary hypertension in neonates, NO has been administrated to invasively ventilated patients suffering from ARDS. Several studies sponsored by NO manufacturers have analyzed NO gas cylinder therapy in ARDS. Inhaled NO has shown in randomized controlled trials a benefit in short-term therapy but not long-term effects. Improvement in oxygenation but no mortality benefits was concluded. However, the complexity of pathologies in children and adults, dosages and co-treatments make difficult the analysis of these different studies [13-15].Variation of the effects of NO treated patients may be due to differences in pulmonary, underlying co-morbidities and potential co-treatments [16].

NO is a key regulatory component of many signalling pathways in the body. NO regulates inflammation, immune responses, cell adhesion, vasodilatation, angiogenesis, neurotransmission and its dysregulation has been associated with many pathologies including inflammatory disorders and cancer[17].NO signals by several pathways including S-Nitrosylation and guanylate cyclase activation and mitogen-activated protein kinase [17, 18]. NO plays a role in host defence mechanisms as deficient NO synthase mice have been shown to be unable to fight and survive mycobacterial infection and influence cytokine production [19].

Inhalation of NO has been used for the treatment of severe acute respiratory syndrome caused by a rapidly spreading corona virus (SARS-CoV) appeared in China in 2002. Chen et al showed in one study with a small group of patients improvement of arterial oxygenation allowing shortened the length of ventilator support as compared to control patients[20]. A direct effect of NO donors on viral replication was also reported indicating a larger effect of NO [21].

With the recent outbreak of COVID-19, inhaled NO has been proposed as an interventional rescue therapy[22]. Clinical studies are going to define the efficacy of inhaled NO in COVID-19. Two studies with a small group of patients in intensive care units under ventilation did not showed improvement with short NO treatment (15 to 30 minutes) [23, 24]. Large studies should define if NO can be effective as emergency therapy for COVID-19 and better determine the group of patients that can benefit of this therapy[25].

In conclusion, our study shows that a low-cost and portable device generating NO without limitation that has previously been used in clinical studies to treat neonate pulmonary hypertension is now tested in LPS-induced ARDS showing an attenuation of pulmonary inflammation in mice. We hope to extend the benefit of NO to populations that do not have access to this therapy today.

Conflicts of interest

The authors declare that they have no conflict of interests.

Author's contribution

All authors contributed to data collection and analysis and have reviewed and approved the final manuscript. IG, LC, IM, PC, and LCG contributed to the design of the project, animal and clinical studies and analysis of data. IG, LC, IM, PC, LCG and BR contributed to data generation analysis, and preparation of the final manuscript.

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