Short Communication

Inhaled nitric oxide decreases the bacterial load in a rat model of *Pseudomonas aeruginosa* pneumonia

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Abstract

Gaseous nitric oxide (NO) is bactericidal *in vitro*. However whether and how it can be used for the treatment of bacterial lung infections in patients with cystic fibrosis is unclear. Here we assessed the bactericidal effect of intermittently inhaled 160 ppm NO for 30 min every 4 h in a *Pseudomonas aeruginosa* pneumonia model in rats. NO significantly reduced *P. aeruginosa* colony count in rat lungs but did not affect neutrophil myeloperoxidase function methemoglobin percentage nor plasma nitrite/nitrate levels. This regimen warrants exploration in infected patients with cystic fibrosis.
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1. Introduction

Cystic fibrosis (CF) is linked to mutations in the epithelial chloride channel, named CF transmembrane conductance regulator (CFTR) and thus have difficulty mobilizing pulmonary secretions. Consequently, microbial infections still represent the most serious clinical complication in CF patients [1]. Antibiotic treatment, has considerably improved the quality of life and mortality in individuals with CF, especially for those patients with infections caused by *Pseudomonas aeruginosa*, the dominant infecting organism in the majority of adult CF patients [2]. However, *P. aeruginosa* as with other microbial pathogens, tend to develop resistance to various antibiotics in CF patients. This is exacerbated when the pathogens in chronic infective states cannot be eradicated and patients are treated with repeated aggressive therapeutic courses [2,3]. Thus, alternative antimicrobial treatment strategies need to be developed.

Nitric oxide (NO) affects many physiologic processes [4] and due to its beneficial effect as a selective pulmonary vasodilator for pulmonary hypertension NO has been studied as a therapeutic gas at concentrations of 20–40 ppm for the treatment of acute respiratory distress syndrome (ARDS) [5] and is already approved in full-term newborns with persistent pulmonary hypertension [6].

Endogenous NO also contributes to host defenses against a wide range of bacteria, fungi and viruses upon up-regulation of neutrophils, macrophages or epithelial cells following infection and/or stimulation by cytokines [7–11]. The microbicidal effect of NO is thought to be mediated by interference with DNA repair mechanisms, binding to heme containing or thiol

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containing metabolic proteins, damaging membrane lipids, or modulating the host immune response [7,9–11]. These data warrant the exploration of inhaled exogenous NO for the treatment of respiratory infections in CF patients; however, little *in vivo* data for an optimal treatment strategy are available.

Antimicrobial effects of exogenous NO have been demonstrated *in vitro* [12–14] and in a rat model of acute *P. aeruginosa* pneumonia [15]. In the latter study, NO was administered continuously at 40 ppm. Based on our *in vitro* data [14], we hypothesized that intermittent inhalation of 160 ppm would be comparably beneficial *in vivo*. Here we report that intermittent high dose NO treatment of rats induced with *P. aeruginosa* pneumonia resulted in a significant reduction of *P. aeruginosa* bacterial load expressed as colony-forming units (CFUs) in the airways of the rats. This inhaled antimicrobial NO regimen did not adversely affect lung inflammation nor did it result in toxic nitrite/nitrate nor methemoglobin blood levels, suggesting that this treatment regimen may represent a promising therapeutic approach for patients with bacterial lung infections.

2. Material and methods

Male Sprague–Dawley rats (300–450 g body weight, Charles River Labs, St. Constant, Quebec, Canada) were anesthetized with 5% halothane in 100% oxygen and challenged intratracheally with 0.2 mL of 1.2×10^8 CFU of *P. aeruginosa* (ATCC strain 27853) or 0.2 mL sterile phosphate buffered saline (PBS), pH 7.4, as previously described [15]. Thereafter, groups of uninfected animals (n=6) and *P. aeruginosa* infected animals (n=20) were treated with room air ($FiO_2 = 0.21$, NO < 0.5 ppm) or 160 ppm NO in room air for 30 min every 4 h for 12 h or 24 h. Animals in the NO treatment groups were continuously monitored in a Plexiglas chamber, flushed at 10 L/min with a gas mixture of NO in nitrogen (800 ppm, ViaNOx-H, VIASYS Healthcare, Yorba Linda, CA, USA), medical-grade air ($FiO_2 = 0.21$, Praxair, Mississauga, Ontario, Canada) and medical-grade oxygen to maintain $FiO_2 = 0.21$ (Praxair, Mississauga, Ontario, Canada) using high-accuracy flow meters and a custom gas control manifold (Pulmonox Medical Inc., Tofield, Canada). A fan driven soda lime (Sodasorb, WR Grace Company, Cambridge, MA. USA) absorber module attached to the Plexiglas chamber circulated the gas in the chamber to remove the CO_2 produced by the rats as well as the NO_2 product of the reaction of NO with oxygen. The gas mixture was monitored continuously by an electrochemical-based NO, NO_2 and O_2 analyzer (Aero Nox, Pulmonox Medical Inc., Tofield, Canada).

Half of the animals were euthanized after 12 h and the balance at 24 h (1 mL/kg of 2.5% sodium thiopental, intravenously). Heparinized arterial blood was collected for determination of NO metabolites [16] and methemoglobin percentage (ABL 3, Radiometer, Copenhagen, Denmark). Lungs were removed *en bloc*, the right lung weighed and homogenized (Polytron homogenizer, Brinkmann, Mississauga, Ontario, Canada) in 3 mL of sterile sucrose (0.32 M) dissolved in HEPES buffer (10 mM, pH 7.4; Sigma, St. Louis, MO, USA) for assessment of total pulmonary bacterial load and neutrophil-derived myeloperoxidase (MPO) activity using routine methods [16]. The study protocol was approved by the University of Western Ontario Animal Care and Use Committee and was carried out under the supervision of a veterinarian in accord with international principles on the care and handling of animals in research as mandated by the Canadian Council on Animal Care. For statistical analysis the two-tailed Student's t-test employing a significance level of p < 0.05 was used.

3. Results and discussion

Lungs from infected animals treated with room air, appeared grossly edematous with multifocal areas of hemorrhage (not shown). High counts of *P. aeruginosa* CFUs were present at 12 and 24 h after bacterial challenge (Fig. 1). Intermittent inhalation of 160 ppm NO to infected rats was associated with a significantly reduced bacterial load at these time points (Fig. 1). Our findings are consistent with previous data demonstrating a modest antibacterial efficacy of continuous exposure of 40 ppm inhaled NO in rats with *P. aeruginosa* pneumonia [12]. *In vitro* data suggest that higher concentrations of NO administration are directly bactericidal, while low levels of NO administration may exert antibacterial effects indirectly *via* stimulation of the host immune system [13].

Although intermittently inhaled NO at concentrations of 160 ppm may be effective in treating human respiratory infections, NO itself may be toxic resulting in increased inflammation or inducing methemoglobinemia directly or *via* other NO metabolites such as peroxynitrite. We therefore measured pulmonary MPO activity, a marker of leukocyte infiltration in rat lung homogenates. MPO activity increased significantly as a consequence of pneumonia in room air treated rats at 24 h (Fig. 2). The addition of NO treatments for up to 24 h did not affect these MPO levels significantly (Fig. 2). In addition, we monitored plasma nitrite/nitrate levels. These results were consistent with previous reports [15], demonstrating that plasma nitrite/nitrate levels continuously
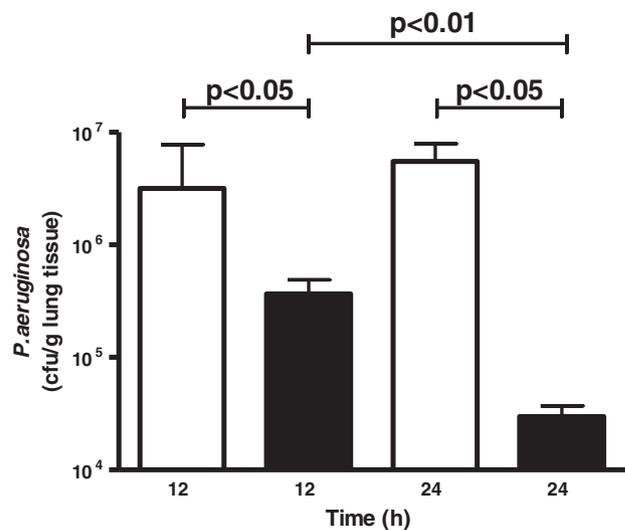

Fig. 1. Effect of high-dose intermittent NO inhalation on *Pseudomonas aeruginosa* cell numbers in a lung infection model in rats. Solid bars represent treatment (160 ppm NO for 30 min every 4 h) and open bars represent control (continuous room air for 12 or 24 h).
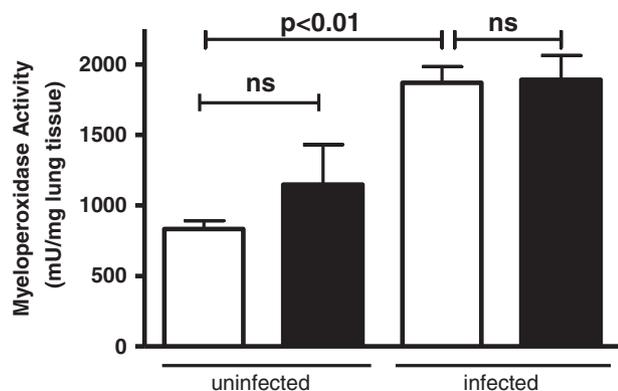
Fig. 2. Myeloperoxidase activity in infected and un-infected rats, treated with high-dose intermittent NO. The influx of neutrophils into rat lung tissues was measured in *P. aeruginosa* infected and uninfected (PBS) rats. Solid bars represent treatment (160 ppm NO for 30 min every 4 h) and open bars represent control (continuous room air for 24 h). ns=statistically not significant.

increased in uninfected rats due to NO treatment by 24 h (Fig. 3). Interestingly, the nitrite/nitrate level also increased in room air treated rats with infection. Presumably this was due to increased endogenous NO upregulation caused by the presence of the bacterial infection alone (Fig. 3) This has been previously reported in human patients with pulmonary infections [17]. Importantly, the infected rats treated with NO did not result in further increases in plasma nitrite/nitrate levels (Fig. 3).

Finally, blood methemoglobin percent levels did not differ between the 4 rat groups at 24 h and were all less than 1% (not shown). Methemoglobin is formed in direct relation to the concentration of NO and the duration of NO inhalation and rises to 1% following 30 min of breathing 160 ppm NO. Once formed, methemoglobin is reduced to hemoglobin by methemoglobin reductase. Thus, the predicted metabolic half-life of methemoglobin is approximately 1 h in humans [18], and likely to be similar in rats. The baseline methemoglobin percent levels were measured in this study and also 3.5 h after the last
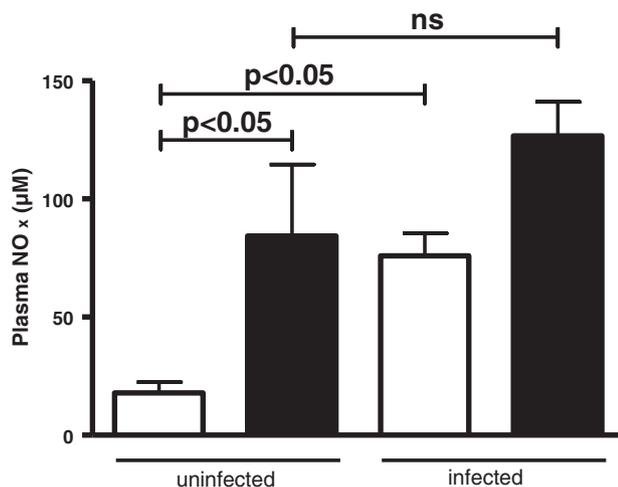

Fig. 3. Plasma nitrite/nitrate levels in *P. aeruginosa* infected rats treated with high-dose intermittent NO. Nitrite/nitrate (NOx) levels were determined in plasma samples of *P. aeruginosa* infected and uninfected (PBS) rats. Solid bars represent treatment (160 ppm NO for 30 min every 4 h) and open bars represent control (continuous room air for 24 h). ns=statistically not significant.

exposure of the rats to 160 ppm NO. Similar baseline methemoglobin percent values and other safety parameters such as lung function and inflammatory markers have been determined in a recent phase I study of intermittent 160 ppm NO treatment in 10 healthy human individuals [19]. We have shown that intermittent high dose NO treatment in rats with a pulmonary infection appears to be safe and effective. The intermittent schedule of high dose NO treatment may be very practical from a clinical perspective due to the freedom of movement between treatment courses in CF patients with respiratory infections. Taken together, with the healthy human and these data provide sufficient rationale to explore this antimicrobial regimen in humans with pulmonary infections. However, whether such a treatment regimen is equally effective in CF patients as in our rat study is not yet known. The data suggests that further studies in CF patients are warranted.

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