Effects of Nebulized Diethylenetetraamine-NONOate in a Mouse Model of Acute Pseudomonas aeruginosa Pneumonia*

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Objective: Endogenous and exogenous nitric oxide (NO) may have important antibacterial effects in patients with pneumonia. NO administration has been limited to the continuous inhalation of gas-phase NO (i.e., inhaled NO [iNO]). Intermittent nebulization of NONOates, novel NO donors, may permit the continuous intrapulmonary delivery of NO. Thus, we assessed the effects of nebulized diethylenetetraamine-NONOate (DETA-NO) in a model of acute Pseudomonas aeruginosa pneumonia.

Design: Randomized, controlled study.

Subjects: Male C57Bl/6 mice.

Interventions: Pneumonia was induced by intratracheal instillation of Pseudomonas aeruginosa (3 × 10^7 CFU in 50 μL). Pneumonia and sham mice were randomized to receive no treatment, nebulized DETA-NO (12.5 or 125 μmol) at 4 h and 12 h, or continuous iNO for 24 h (10 or 40 ppm) until they were killed at 24 h.

Main results: The nebulization of DETA-NO was associated with a marked increase in mean (± SEM) exhaled NO levels (after nebulization, 484 ± 34 parts per billion [ppb]; baseline, 13.4 ± 0.4 ppb; p < 0.01) and plasma levels of nitrites/nitrates (after nebulization, 73 ± 28 μM; at baseline, 14 ± 3 μM; p < 0.05). Nebulized DETA-NO decreased the pulmonary bacterial load in mice with pneumonia by 65 ± 19% (p < 0.05 vs untreated mice) but had no effect on pulmonary leukocyte infiltration. Although the growth of P. aeruginosa colonies in vitro was impaired on exposure to DETA-NO, growth was similarly impaired by exposure to DETA nucleophile/backbone alone.

Conclusions: The nebulization of DETA-NO provides a method for the prolonged intrapulmonary delivery of NO. The antibacterial effect of DETA-NO in vivo and in vitro is due, in large part, to the DETA nucleophile moiety and is independent of NO, suggesting a limited therapeutic role for exogenous NO in pneumonia. (CHEST 2002; 122:2127–2136)

Key words: exhaled nitric oxide; inhaled nitric oxide; lung inflammation; nitric oxide; nitric oxide donors; nitrites/nitrates

Abbreviations: ANOVA = analysis of variance; DETA-NO = diethylenetetraamine-NONOate; eNO = exhaled nitric oxide; iNO = inhaled nitric oxide; MPO = myeloperoxidase; NO = nitric oxide; NOx = nitrites/nitrates; PBS = phosphate-buffered saline solution; RA = room air

Bacterial pneumonia remains a common and important clinical problem. Despite effective antimicrobial therapy, pneumonia has significant morbidity and mortality, especially in hospitalized patients and in those patients with certain pathogens, such as Pseudomonas aeruginosa.1,2 Pneumonia is characterized by intrapulmonary bacterial proliferation, pulmonary edema, neutrophil infiltration, and parenchymal lung injury. Therapy targeted at these pathophysiologic features of pneumonia may be beneficial in the clinical management of patients with pneumonia.

Nitric oxide (NO) is a ubiquitous mammalian mediator with many homeostatic effects, such as vasodilation and immune modulation. Endogenous NO has been shown to have both proinflammatory and anti-inflammatory effects in various models of...
lung injury. Moreover, the antibacterial effects of NO also have been described.3–5

The gaseous nature of NO and its recognized selective pulmonary vasodilatory effects have led to the clinical use of inhaled NO (iNO) in pulmonary hypertension and in acute lung injury.6–8 Furthermore, antibacterial and anti-inflammatory effects of iNO in various models of lung injury have been suggested.5,9–11 Continuous iNO therapy usually is restricted to patients who are intubated and ventilated with closed circuits because of concerns about health-care giver safety on exposure to ambient NO and its oxidative metabolite, NO2.12,13

NONOates are synthetic adducts of NO and a nucleophile backbone that spontaneously and nonenzymatically release NO at predictable rates.14–17 Different NONOates are characterized by differences in half-life and the resulting rate of NO release. The intermittent inhalation of a nebulized NONOate solution may permit the continuous release of NO in the lower respiratory tract, which may avoid the technical complexities and potential risks of continuous iNO delivery.

Thus, we hypothesized that nebulized diethylenetetraamine-NONOate (DETA-NO), through intrapulmonary NO release, would have beneficial antibacterial and anti-inflammatory effects in a mouse model of acute P aeruginosa pneumonia.

**Materials and Methods**

**Murine Model of P aeruginosa Pneumonia**

The experimental protocol was approved by our institutional Animal Care and Use Committee, in accord with the guidelines of the Canadian Council on Animal Care, and was supervised by a veterinarian. Male C57Bl/6 mice (weight range, 20 to 25 g; age, 6 to 7 weeks; Charles River Laboratories; St. Constant, PQ, Canada) were allowed at least a 48-h period of environmental acclimatization before experimentation and were provided access to food and water ad libitum.

An established rat model of P aeruginosa pneumonia, previously described by ourselves,16 was adapted to the mouse. Under general anesthesia (halothane in oxygen) and aseptic conditions, an anterior tracheotomy was performed with a 24-gauge needle. Mice were randomized to either a pneumonia group (n = 90) or a sham group (n = 40). Pneumonia was induced by intratracheal instillation of a 50-μL aliquot of a suspension of P aeruginosa (approximately 6 × 106 cfu/mL; strain No. 27853; American Type Culture Collection; Manassas, VA) followed by a 200-μL bolus of air. Animals in the sham group underwent anesthesia and tracheostomy but did not undergo intratracheal instillation.

Mice were allowed to recover in cages with access to fluid and water ad libitum and were monitored for 24 h until they were killed. During this time, the mice received a total of 6 mL grade air at a final chamber NO concentration of either 10 or 40 ppm, respectively. In brief, spontaneously breathing mice were individually placed in a small plastic chamber that was continuously flushed with medical-grade air at 60 mL/min. The chamber effluent, containing exhaled gas, was sampled in triplicate, and the concentration of gas-phase NO measured by chemiluminescence. Samples were referenced to a calibration curve of signal mV vs standard concentrations of gas-phase NO (0 to 527 parts per billion; R2 ≥ 0.999). Data are reported as the concentration of eNO in parts per billion.

DETA-NO Preparation and Delivery

DETA-NO was chosen because it has the longest half-life of commercially available NONOates (56 h at 22°C in a 0.1 M phosphate buffer at pH 7.4).18 Solutions of DETA-NO in PBS were prepared fresh immediately before nebulization. For exposure to DETA-NO, mice were housed in a 5-L plastic (Plexiglas; Rohm and Haas; Philadelphia, PA) chamber into which 2.5 mL DETA-NO/PBS were completely nebulized over 10 min (LC plus jet-nebulizer; PARI Respiratory Equipment, Inc; Mississauga, ON, Canada; and Medi-mist compressor, model 1802; Mountain Medical Equipment; Littleton, CO). The estimated average chamber DETA-NO concentration during nebulization would be approximately 1.0 μmol/L and approximately 10 μmol/L for the 12.5 and 125 μmol doses, respectively. Given that two molecules of NO are released by each molecule of DETA-NO, these DETA-NO chamber concentrations are equivalent to iNO levels of approximately 40 ppm and approximately 400 ppm, respectively.

In pilot studies, the intrapulmonary release of NO was assessed following a single exposure to 125 μmol nebulized DETA-NO (4 h after surgery) in animals in both the pneumonia and sham groups. Separate groups of animals were sacrificed before and at several time points (ie, 0.5, 1.5, 2.5, and 12 h) after DETA-NO exposure for the measurement of plasma and BAL levels of nitrites/nitrates (NOx−). At each time point, mice were killed, blood was aspirated via cardiac puncture, and a tracheostomy was performed with a 24-gauge catheter. BAL was carried out by instilling and withdrawing a single 1-mL aliquot of PBS three times.

In a subset of animals, exhaled NO (eNO) levels were serially measured noninvasively before and 0.5, 2, 6, 12, and 24 h after the single DETA-NO nebulization, as previously described.20 In brief, spontaneously breathing mice were individually placed in a small plastic chamber that was flushed with NO-free medical-grade air at 60 mL/min. The chamber effluent, containing exhaled gas, was sampled in triplicate, and the concentration of gas-phase NO measured by chemiluminescence. Samples were referenced to a calibration curve of signal mV vs standard concentrations of gas-phase NO (0 to 527 parts per billion; R2 ≥ 0.999). Data are reported as the concentration of eNO in parts per billion.

In order to assess the effects of DETA-NO on pulmonary bacterial burden and inflammation, mice in both the sham and pneumonia groups were twice exposed (at 4 and 12 h) to DETA-NO during the 24-h experimental protocol.

**Exposure to iNO**

During the 24-h protocol, mice were housed in a sealed plastic chamber that was continuously flushed with NO gas in medical-grade air at a final chamber NO concentration of either 10 ± 1 or 40 ± 2 ppm, as continuously measured by chemiluminescence (Pac III NO analyzer; Drager; Mississauga, ON, Canada). Chamber NO2 concentrations were intermittently monitored and were consistently < 2 ppm. Mice exposed to RA were housed in an identical plastic chamber continuously flushed with medical-grade air (final chamber NO concentration, < 0.3 ppm) for 24 h.
Pulmonary Leukocyte Infiltration and Total Pulmonary Bacterial Load

Twenty-four hours following surgery, animals were killed with 0.5 mg/g body weight of intraperitoneal pentobarbital (65 mg/mL). Blood was collected in a heparinized syringe via cardiac puncture. Heparinized blood samples were centrifuged at 10,000g for 10 min at 4°C, and the plasma supernatant was collected and stored under nitrogen at −20°C until assayed for NOx levels (vide infra). The heart and lungs were removed en bloc, and the pulmonary vasculature was perfused with 10 mL saline solution through a right ventriculotomy. Lung tissue was homogenized in 1 mL 10 mM bicarbonate-free N-2-hydroxyethylpiperazine-N’-2-ethanesulfonic acid buffer (containing 0.1 mM ethylenediaminetetraacetic acid, 1 mM MgCl2, phenylmethylsulfonil fluoride, 1 mM dithiothreitol, and 0.32 M sucrose [pH 7.4]) at 4°C for analysis of myeloperoxidase (MPO) content and pulmonary bacterial load.

Pulmonary leukocyte infiltration was assessed by the measurement of MPO activity, as previously described. Briefly, solubilized MPO activity was assessed in quadruplicate in a sonicated lung homogenate by assessing the H2O2-dependent oxidation of tetramethylenediamine for > 15 min at 37°C. One unit of MPO activity was defined as a change of 1.0 optical density units at 655 nm/min and was expressed as total pulmonary MPO in milliunits.

The pulmonary bacterial load was determined as previously described. Briefly, a lung homogenate was serially diluted in a sterile 0.9% saline solution, and aliquots were incubated in duplicate on sheep blood agar plates at 37°C for 24 h. The total pulmonary bacterial load was calculated and expressed as a multiple of the instilled number of bacteria. The consistency of the bacterial load in the mice with pneumonia on each experimental day was ensured by twice quantitating the concentration of the P aeruginosa suspension (approximately 6 × 10^7 cfu/mL) that had been used for inducing pneumonia, both before the first mouse and after the last mouse had undergone intratracheal instillation. The coefficient of variation for within-day variability of the bacterial suspension concentration was ≤ 2%.

Analysis of Plasma NO Metabolites (Nitrites and Nitrates)

Plasma levels of nitrites and nitrates were collectively measured as NOx by chemiluminescence, as previously described. Briefly, plasma was refluxed in saturated vanadium chloride in hydrochloric acid, resulting in the reduction of NOx to NO, which is detected by chemiluminescence (model 270B NO analyzer; Sievers Instruments; Boulder, CO). The analyzer was calibrated daily, and samples were referenced to a standard curve generated from NO3− standards (0.05 to 500 μM; R² ≥ 0.999).

Histology

An experienced pulmonary pathologist blindly evaluated the morphologic changes in various experimental groups. In a subset of 26 animals (sham group mice, 3; pneumonia mice that were untreated, 3; and pneumonia mice that were treated with DETA-NO and 13 with iNO), one lung was harvested and fixed for 24 h in buffered formalin under constant vacuum-induced inflation at 20 cm H2O. Representative lung sections were examined for a variety of histologic parameters including atelectasis, vascular congestion, hemorrhage, alveolar edema, accumulation of inflammatory cells, and the presence of bacteria. The distribution of the above changes was assessed as either diffuse (uniform) or patchy (nonuniform). Using a semi-quantitative scoring system, the degree of bronchopneumonia was graded as follows: 0, normal; 1, mild pneumonia; 2, moderate pneumonia; and 3, severe pneumonia.

Results

Mouse Model of Acute P aeruginosa Pneumonia

Following the induction of pneumonia, mice appeared lethargic and tachypneic, and exhibited decreased grooming and exploratory behavior. The lungs of mice with pneumonia were grossly edematous and had patchy areas of hemorrhage. Survival at 24 h was not significantly different in pneumonia mice vs sham mice (p = 0.10) [Table 1]. The inhalation of NO or exposure to nebulized DETA-NO did not have any qualitative effects on the behavior either of sham or pneumonia mice, nor were there any gross pathologic pulmonary effects. Moreover, treatment with iNO or DETA-NO had no significant effect on survival in either pneumonia or sham mice (Table 1).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Sham Mice Survived/Total %</th>
<th>Pneumonia Mice Survived/Total %</th>
</tr>
</thead>
<tbody>
<tr>
<td>None</td>
<td></td>
<td>19/19</td>
<td>28/32</td>
</tr>
<tr>
<td>DETA-NO</td>
<td>12.5 μmol</td>
<td>7/7</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>125 μmol</td>
<td>10/10</td>
<td>100.0</td>
</tr>
<tr>
<td>iNO</td>
<td>10 ppm</td>
<td>6/6</td>
<td>100.0</td>
</tr>
<tr>
<td></td>
<td>40 ppm</td>
<td>7/7</td>
<td>100.0</td>
</tr>
</tbody>
</table>

Table 1—Effect of DETA-NO and iNO on Survival of Sham Mice and Mice with P aeruginosa Pneumonia
Effects Of DETA-NO and iNO on Pulmonary Bacterial Load

In untreated pneumonia mice, the total pulmonary bacterial load was 6.1 ± 1.8-fold greater than the instillate bacterial number, indicating intrapulmonary bacterial proliferation over the 24 h following intratracheal instillation (Fig 1). DETA-NO exposure (both 12.5 and 125 μmol) was associated with a 65 ± 19% (p < 0.05) decrease in the total pulmonary bacterial load. There was no difference in the effect on bacterial load of the two doses of DETA-NO. However, the nebulization of 2.5 μmol DETA-NO had no significant effect on pulmonary bacterial load (data not shown). In contrast to DETA-NO exposure, iNO exposure was associated with an increased pulmonary bacterial load at 40 ppm (241 ± 55% of the bacterial load in mice in the untreated pneumonia group; p < 0.05) but not at 10 ppm (Fig 1).

Effects Of DETA-NO and iNO on Pulmonary Neutrophil Infiltration

Pulmonary MPO activity, a marker of leukocyte infiltration, was significantly increased in pneumonia vs sham mice (Fig 2). Pulmonary MPO levels in mice with pneumonia were unaffected either by nebulized DETA-NO or iNO. In sham mice, pulmonary MPO activity was slightly increased by nebulization of 125 μmol DETA-NO, but not 12.5 μmol DETA-NO. In contrast, the low level of pulmonary MPO measured in sham mice was further reduced following exposure to iNO at both 10 and 40 ppm, although only the 40-ppm dose achieved statistical significance.

The morphologic appearance of lungs from sham mice, mice with untreated pneumonia, and mice with pneumonia that were treated (with DETA-NO and iNO) are shown in Figure 3. The lungs of sham mice showed normal morphology except for mild peripheral atelectasis and vascular congestion (grade 0). Compared to sham mice, the lungs of mice with pneumonia were characterized by mild-to-moderate bronchopneumonia with vascular congestion, edema, and bronchiolar/airspace inflammatory infiltrates that were composed of neutrophils (grade 1 to 2). The lungs of mice nebulized with DETA-NO and iNO showed similar histology in terms of neutrophilic infiltration and vascular congestion, with the degree of bronchopneumonia ranging from mild to severe (grades 1 to 3). In severely affected animals, there was extensive vascular congestion, hemorrhage, and neutrophilic infiltration. However, compared to mice with pneumonia that were treated with DETA-NO and untreated mice with pneumonia, iNO exposure was associated with the presence of more abundant bacteria and less alveolar edema.

![Figure 1. Effect of nebulized DETA-NO (12.5 or 125 μmol) or iNO (10 or 40 ppm) on the pulmonary bacterial burden as a multiple of the number of organisms instilled in mice with P aeruginosa pneumonia (untreated pneumonia control [C] group, 28 mice; other treatment groups, 6 to 12 mice per group). * = p < 0.01 for DETA-NO-treated group or iNO-treated group vs the untreated pneumonia group.](http://journal.publications.chestnet.org/pdfffaccess.ashx?url=/data/journals/chest/21985/ on 06/26/2017)
Effects Of DETA-NO and iNO on Endogenous NO Levels

In pilot studies, exposure to a single dose of 125 μmol nebulized DETA-NO was associated with increased eNO and plasma NOx^- levels but no change in NOx^- levels in BAL fluid (Fig 4). eNO levels increased markedly within 30 min, which is consistent with intrapulmonary NO release, and then declined to baseline levels after >24 h. Plasma levels of NOx^- also were markedly increased within 30 min of DETA-NO nebulization and then declined to baseline within 12 h (Fig 4B). Plasma NOx^- levels were significantly greater in mice with untreated pneumonia than in sham mice (Fig 5). Continuous exposure to iNO during the 24 h of the protocol increased plasma NOx^- levels in both sham and pneumonia mice but had a greater effect in mice with pneumonia. Moreover, doses of 10 and 40 ppm had similar effects in sham mice but plasma NOx^- levels were greater following administration of a dose of 40 ppm iNO than of 10 ppm iNO in mice with pneumonia.

When sham mice were twice exposed (at 4 and 12 h) to nebulized DETA-NO (both 12.5 and 125 μmol doses) during the 24-h protocol, there was no effect on plasma NOx^- levels assessed at 24 h vs those in untreated sham mice (Fig 5). In mice with pneumonia that had been exposed to DETA-NO at 4 and 12 h, plasma NOx^- levels at 24 h were slightly reduced after exposure to 125 μmol DETA-NO and were slightly higher after exposure to 12.5 μmol DETA-NO compared to those in mice with untreated pneumonia.

Effects of DETA-NO Exposure on P aeruginosa Growth In Vitro

Exposure to DETA-NO at 5 and 50 mM (which are equivalent to nebulized doses of 12.5 and 125 μmol, respectively) completely inhibited the growth of P aeruginosa, whereas exposure to DETA-NO at 0.5 mM had a lesser inhibitory effect (Table 2). Exposure to the DETA nucleophile alone (ie, exhausted DETA-NO) inhibited bacterial growth at all concentrations, which is similar to DETA-NO. NaN02 had only a slight inhibitory effect on bacterial growth at a dose of 50 mM.

Discussion

Exposure to nebulized DETA-NO, a NO donor, is associated with significant, prolonged intrapulmonary NO release in mice. In a mouse model of P aeruginosa pneumonia, the intermittent nebuliza-
tion of DETA-NO significantly attenuated intrapulmonary bacterial growth. However, a possible in vivo antibacterial effect of exogenous NO was not confirmed during the administration of iNO, which at 40 ppm was associated instead with an increased pulmonary bacterial load. The exposure of P. aeruginosa to DETA-NO in vitro demonstrated a direct antibacterial effect. However, an equipotent antibacterial effect was observed in vitro on exposure to the DETA nucleophile moiety alone, which was generated from DETA-NO following the release of all bound NO. Thus, the in vitro and in vivo antibacterial effects of DETA-NO appear to be localized to the DETA moiety and are largely independent of NO. Neither DETA-NO nor iNO had any effect on pulmonary inflammation in mice with pneumonia.

NO is a multifunctional mediator with important homeostatic vascular and immune effects. Recognition of the physiologic pulmonary vascular effects of endogenous NO led to the development of iNO therapy for conditions such as pulmonary hypertension and acute lung injury. Unfortunately, the delivery of iNO therapy is technically challenging because of concerns about ambient levels and health-care giver exposure. Furthermore, since the effects of iNO are transient and are not sustained upon withdrawal, iNO must be provided on a continuous basis. Thus, there has been intensive research into alternative strategies for the administration of exogenous NO. Several other NO donors exist, including S-nitrosothiols, nitrovasodilators, and NONOates. Most NO donors do not release NO at

**Figure 3.** Hematoxylin and eosin-stained light micrographs of histologic sections from mice lungs. **Top left,** a: lung from a sham mouse showing normal airspaces, interstitium, and bronchioles (original ×90). **Middle left,** b: lung from untreated mouse with pneumonia showing moderate bronchopneumonia with vascular congestion, alveolar edema, and moderate neutrophil infiltration (original ×90). **Bottom left,** c: DETA-NO-treated lung from a mouse with pneumonia showing severe bronchopneumonia with edema and extensive neutrophilic infiltration (original ×90). Lung from a mouse with pneumonia that has been treated with iNO showing moderate bronchopneumonia (top right, d) [original ×220] and abundant bacteria (middle right, e) [original ×350]. The large arrows indicate neutrophils; the small arrows indicate bacteria.
predictable rates, being sensitive to local conditions including pH, redox status, thiol levels, and enzymatic degradation.²³,²⁴

NONOates, adducts of NO and various nucleophile moieties, are distinct NO donors that spontaneously and nonenzymatically release NO at predictable rates.¹⁵,¹⁶,¹⁹,²⁵ The in vivo decomposition studies described in the present report confirm continuous intrapulmonary NO release for at least 12 to 24 h following the exposure of mice to nebulized DETA-NO. Although the mice clearly received a large dose of NO during DETA-NO nebulization, the precise dose remains largely unknown in the absence of actual measurements of DETA-NO concentration in the exposure chamber, the minute ventilation of individual mice, and the physicochemical properties of nebulized DETA-NO. However, given the negative results with iNO exposure, which provided NO in gaseous form at a uniform chamber concentration, the potential factors affecting actual NO delivery by nebulized DETA-NO are less relevant in our model of pneumonia.

Figure 4. Effects of exposure of mice to a single dose of nebulized DETA-NO (125 µmol) on (top) eNO levels in parts per billion (ppb) and (bottom) plasma and BAL NOX⁻ levels (6 to 12 animals per group). * = p < 0.05 (vs respective preDETA-NO baseline); ** = p < 0.01 (vs respective preDETA-NO baseline).
Intrapulmonary delivery of nebulized DETA-NO in solution/liquid is associated with initial release of dissolved, gaseous, free radical NO\(^{18}\) (unpaired electron). Clearly, a portion of this NO was lost in exhaled breath. This free radical also may react with various other free radicals, such as superoxide radical and macromolecules, including thiols, lipids, and peptides.\(^{23}\) The resulting products, including peroxynitrite and S-nitrosothiols, are themselves important biological mediators of both physiologic and inflammatory responses.\(^{26,27}\) Indeed, some of the effects ascribed to NO can instead be mediated by such NO-related species. Regardless of these interactions, the ultimate disposal of endogenous and exogenous NO (eg, iNO or from DETA-NO) is thought to be through oxidative metabolism to nitrites/nitrates (NO\(^x\)). For example, nitrates are the final products of the reaction of NO with intravascular hemoglobin as well as the oxidative decomposition of peroxynitrite (ONOO\(^-\)), through peroxynitrous acid (ONOOH), to the radicals OH\(^-\) and NO\(_2^-\).\(^{28}\) It is notable that despite the significant, sustained elevations of both eNO and plasma levels of NO\(^x\) following exposure to nebulized DETA-NO, the BAL levels of NO\(^-\) remained unchanged. This suggests minimal intrapulmonary oxidation of NO.

When NO\(^x\)ates are administered IV, they have been shown to cause both systemic and pulmonary vasodilation in animals.\(^{14,15,25,29}\) The rate of NO release and the resulting degree of vasodilation are related to the chemistry of the nucleophile moiety of the individual NO\(^x\)ate compounds.\(^{25,30}\) The intratracheal instillation of NO\(^x\)ate has been shown to selectively dilate the pulmonary vasculature both in normal rats and in rats with U-46619-induced pulmonary hypertension.\(^{31}\) Aerosolized NO\(^x\)ate also

**Table 2—In vitro Growth of* P. aeruginosa* During Exposure to DETA-NO, Exhausted DETA-NO, and NaNO\(_2\)**

<table>
<thead>
<tr>
<th>Experimental Treatment</th>
<th>Concentration (\text{mM})</th>
<th>Initial Bacterial Concentration, cfu/mL</th>
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<tbody>
<tr>
<td>Control</td>
<td>100 ± 8</td>
<td>100 ± 2</td>
</tr>
<tr>
<td>DETA-NO</td>
<td>50</td>
<td>0 ± 0(^1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>1 ± 0(^1)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>79 ± 4(^1)</td>
</tr>
<tr>
<td>Exhausted DETA-NO</td>
<td>50</td>
<td>0 ± 0(^1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>7 ± 2(^1)</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>54 ± 8(^1)</td>
</tr>
<tr>
<td>NaNO(_2)</td>
<td>50</td>
<td>68 ± 4(^1)</td>
</tr>
<tr>
<td></td>
<td>5</td>
<td>97 ± 6</td>
</tr>
<tr>
<td></td>
<td>0.5</td>
<td>72 ± 13</td>
</tr>
</tbody>
</table>

*Values given as mean % survival ± SEM of three experiments.

\(^{1}p < 0.01\) vs control.

\(^{2}p < 0.05\) vs control.
has been shown to attenuate pulmonary hypertension and improve oxygenation in several animal models.\textsuperscript{32,33}

The effects of NONOates have been presumed to be NO-mediated. However, the majority of studies of the effects of NONOates have not assessed the direct effects of the respective nucleophile-backbone moieties. Indeed, in one study\textsuperscript{32} the lack of increase in plasma NOx\textsuperscript{−} levels following nebulized NONOate may argue against the observed pulmonary vasodilator effect being NO-dependent. Although an antibacterial effect of DETA-NO was demonstrated both \textit{in vivo} and \textit{in vitro} in the present study, it is clear that this effect was not necessarily NO-mediated. Indeed, significant antibacterial activity appears to reside in the DETA nucleophile moiety. Similarly, it is conceivable that some of the previously reported effects of various NONOates also may be NO-independent and may be related to the unstudied effects of the various nucleophile moieties.

\textit{In vivo} assessment of the effects of nebulization of the DETA moiety itself may have been of interest, but access to a commercial source of DETA proved difficult and costly. Moreover, during the process used to generate exhausted DETA \textit{in vitro} from DETA-NO, the released NO yielded a significant amount of soluble nitrite. Thus, the nebulization of this exhausted DETA-NO solution, as an \textit{in vivo} control for DETA-NO exposure, would have been associated with the concomitant exposure of mice to significant levels (\textit{i.e.}, 5 to 50 mM) of nitrite. It has been recognized\textsuperscript{34} that peroxidase activity, in the presence of nitrite, can oxidize tyrosine to generate 3-nitrotyrosine. This suggests that nebulized nitrite exposure in mice with pneumonia-induced pulmonary inflammation could result in intrapulmonary NO-dependent oxidative (nitrosative) effects. However, as DETA-NO and exhausted DETA were equally antibacterial \textit{in vitro}, and as NaN\textsubscript{3}O\textsubscript{2} had a minimal effect, it is likely that NO and nitrite derived from DETA-NO did not play an important role in the observed \textit{in vivo} effects of DETA-NO in murine pneumonia.

The exposure of sham mice to high-dose (\textit{i.e.}, 125 \textmu M) DETA-NO was associated with a significant, albeit slight, increase in pulmonary inflammation. This proinflammatory effect may be due to NO itself or to the DETA nucleophile moiety. Indeed, the lack of attenuation of leukocyte infiltration in DETA-NO-exposed mice with pneumonia, despite the decreased pulmonary bacterial load, may be due to a coincident proinflammatory effect of NO or DETA. The proinflammatory effects of NO have been well-recognized.\textsuperscript{26,35,36} NO may contribute to tissue inflammation and cell injury through several mechanisms. One of the most important mecha-

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