Protection from Lipopolysaccharide-induced Lung Injury by Augmentation of Airway S-Nitrosothiols

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Rationale: S-Nitrosothiols (SNO) inhibit immune activation of the respiratory epithelium and airway SNO levels are decreased in inflammatory lung disease. Ethyl nitrite (ENO) is a gas with chemical properties favoring SNO formation. Augmentation of airway SNO by inhaled ENO treatment may decrease lung inflammation and subsequent injury by inhibiting activation of the airway epithelium.

Objectives: To determine the effect of inhaled ENO on airway SNO levels and LPS-induced lung inflammation/injury.

Methods: Mice were treated overnight with inhaled ENO (10 ppm) or air, followed immediately by exposure to aerosolized LPS or saline. Parameters of inflammation and lung injury were quantified 1 hour after completion of the aerosol exposure and correlated to lung airway and tissue SNO levels.

Measurements and Main Results: Aerosolized LPS induced a decrease in airway and lung tissue SNO levels including S-nitrosylated NF- κ B. The decrease in lung SNO was associated with an increase in lung NF- κ B activity, cytokine/chemokine expression (keratinocyte-derived chemokine, tumor necrosis factor- α , and IL-6), airway neutrophil influx, and worsened lung compliance. Pretreatment with inhaled ENO restored airway SNO levels and reduced LPS-mediated NF- κ B activation thereby inhibiting the downstream inflammatory response and preserving lung compliance.

Conclusions: Airway SNO serves an antiinflammatory role in the lung. Inhaled ENO can be used to augment airway SNO and protect from LPS-induced acute lung injury.

Keywords: ethyl nitrite; NF- κ B; nitric oxide; S-nitrosylation

Despite an annual incidence in the United States of approximately 200,000 cases and a mortality rate between 40% and 50%, the pathophysiology of acute lung injury (ALI)/acute respiratory distress syndrome (ARDS) remains poorly understood, with treatment limited primarily to supportive care (1). Nitric oxide (NO) is known to play an important role in regulating the inflammation that is associated with lung injury, and inhaled NO has been used therapeutically in ALI/ARDS to improve ventilation-perfusion mismatch (2). However, inhaled NO treatment does not alter mortality in ALI/ARDS and, in fact, its use may actually exacerbate lung damage because of increased formation of injurious reactive nitrogen species (RNS) (3).

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AT A GLANCE COMMENTARY

Scientific Knowledge on the Subject

S-Nitrosothiols are endogenously produced, antiinflammatory compounds that are present in the lung airway and are known to be deficient in inflammatory lung disease.

What This Study Adds to the Field

Increasing airway S-nitrosothiol levels by treatment with inhaled ethyl nitrite inhibits the pulmonary inflammatory response to lipopolysaccharide. Ethyl nitrite may have therapeutic value in the prevention and treatment of acute lung injury and other inflammatory lung diseases.

S-Nitrosothiols (SNO) are bioactive NO compounds found in high concentration in the lung and, unlike NO, have minimal reactivity with oxygen and superoxide ($O_2^{\cdot-}$), thereby limiting RNS formation (e.g., peroxynitrite) (4). SNO also possesses potent antiinflammatory properties acting primarily through the inhibition of immune response pathways via protein S-nitrosylation (5). One mechanism by which SNO modulates the immune response is by decreasing activation of the transcription factor nuclear factor- κ B (NF- κ B), which controls the expression of numerous acute-response genes (6). Airway SNO levels are known to be decreased in inflammatory lung disease (7, 8), in which NF- κ B activation in both the respiratory epithelium and airway macrophages is required to initiate the downstream inflammatory response (9, 10), suggesting that airway SNO regulation is an important factor in disease pathogenesis.

Ethyl nitrite (ENO) is a gas with chemical properties favoring reaction with thiol to induce SNO formation (11). Inhaled ENO is superior to NO in augmenting intracellular SNO formation *in vitro* (11) and airway SNO *in vivo* with minimal associated RNS production (12). However, although inhaled ENO has been shown to be effective in restoring SNO-mediated vasodilation in pulmonary hypertension (13, 14), and is an antiinflammatory agent in hyperoxia-impaired neonatal lung development (12), its therapeutic value in ALI/ARDS has not been investigated.

In the present study, we examine the effects of pretreatment with inhaled ENO in a mouse model of LPS-induced lung injury (15). We found that LPS exposure led to an acute decrease in airway SNO. Inhaled ENO treatment augmented SNO levels, which correlated with diminished airway neutrophil influx, inflammatory cytokine/chemokine expression, and NF- κ B activation. These results further support an antiinflammatory role for SNO in the lung airway and suggest that inhaled ENO may be beneficial in the treatment of ALI/ARDS. Some of the results of these studies have been previously reported in the form of an abstract (16).

METHODS

Materials

Reagents were from Sigma-Aldrich (St. Louis, MO) unless otherwise specified. For gas sources and description, *see* the online supplement.

Animal Exposure

Six- to 8-week-old, male C57BL/6J mice (Jackson Laboratories, Bar Harbor, ME) were used in the study. Procedures were approved by the institutional animal care and use committee. Animals were exposed overnight (approximately 15 h) to air (21% O₂/N₂) or ENO (10, 25, or 100 ppm) in 21% O₂ with N₂ as the balance gas. Aerosolized LPS (O111:B4 *Escherichia coli* LPS, 4 µg/m³ × 2.5 h) or saline was administered according to a previously described protocol (15). Mice were killed by CO₂ narcosis 1 hour after completion of the aerosolization.

Lung Samples

Whole lung lavage (bronchoalveolar lavage fluid [BALF]) and lung tissue extraction were performed as previously described (15). The lungs were either snap frozen in liquid N₂ for preparation of tissue homogenates or inflation fixed *in situ* at 25 cm H₂O with 4% paraformaldehyde for later histological analysis. Cell counts of the pooled lung lavage fluid were made with a hemocytometer and cell differentials were determined on stained Cytospin preparations. The BALF was centrifuged at 1,500 × *g* for 10 minutes to pellet cells and the protein concentration of the cell-free BALF was determined by the bicinchoninic acid method (Pierce Biotechnology, Rockford, IL). The supernatant samples were used immediately or stored at -80°C for later SNO and cytokine analysis.

SNO Measurements

SNO was quantified in cell-free BALF and freshly homogenized mouse lung tissue (MLT) extracts (*see* the online supplement) by mercury-coupled, photolysis-chemiluminescence detection as previously described (12). Freshly prepared *S*-nitrosoglutathione (GSNO) was used to generate a standard curve. Measurements were made in triplicate.

A biotin switch assay to detect *S*-nitrosylated NF-κB p65 was performed on freshly prepared MLT homogenates as previously described (17).

3-Nitrotyrosine

An ELISA-based kit (Northwest Life Science Specialties, Vancouver, WA) was used to quantify 3-nitrotyrosine (3-NT) protein levels in BALF.

3-NT immunoreactivity was assessed in representative lung sections of mice exposed to LPS or saline with or without inhaled ENO treatment, using a polyclonal rabbit anti-3-NT antibody (Upstate Cell

Figure 1. Effect of inhaled ethyl nitrite (ENO) on airway *S*-nitrosothiols (SNO). Shown is the SNO concentration in mouse bronchoalveolar lavage fluid after a 15-hour exposure to ENO (0, 10, 25, and 100 ppm). *n* = 5 per group; means + SEM; **P* ≤ 0.05, #*P* ≤ 0.01 versus control.

Figure 2. Effect of aerosolized lipopolysaccharide (LPS) and inhaled ethyl nitrite (ENO) on lung *S*-nitrosothiols (SNO). (A) SNO concentration in bronchoalveolar lavage fluid 1 hour after completion of aerosolized LPS ± inhaled ENO (10 ppm). *n* = 5 per group; mean + SEM; **P* < 0.01 LPS versus saline, LPS + ENO versus saline + ENO, LPS alone; ***P* < 0.05 saline + ENO versus saline alone. (B) SNO concentration in mouse lung tissue (MLT) 1 hour after completion of aerosolized LPS ± inhaled ENO (10 ppm). *n* = 5 per group; mean + SEM; ***P* < 0.05 saline versus LPS, saline + ENO; **P* < 0.01 LPS + ENO versus saline + ENO. (C) *S*-Nitrosylation of NF-κB p65 (SNO-p65) assessed by biotin switch assay in MLT 1 hour after completion of aerosolized LPS ± inhaled ENO (10 ppm). (D) Densitometric analysis of ratio of SNO-p65 to total p65 in MLT 1 hour after completion of aerosolized LPS ± inhaled ENO (10 ppm). Solid columns = LPS; open columns = saline; *n* = 3 per group; mean + SEM; **P* < 0.01 saline versus LPS, saline + ENO; ***P* < 0.05 LPS + ENO versus LPS alone.

Signaling Solutions, Lake Placid, NY) followed by peroxidase detection with diaminobenzidine substrate (Vector, Burlingame, CA). Hematoxylin was used as a counterstain.

Cytokine Analysis

A fluorescent bead immunoassay (Bio-Plex; Bio-Rad Laboratories, Hercules, CA) was used to quantify the concentrations of murine tumor necrosis factor (TNF)- α , keratinocyte-derived chemokine (KC), IL-6, and IL-10 in BALF. Samples were measured in duplicate in five animals per treatment group.

NF- κ B Activity Assays

Nuclear proteins were extracted from frozen lung tissue according to a previously described method (18) and NF- κ B DNA binding was quantified with an ELISA-based kit (TransAm p65; Active Motif, Carlsbad, CA).

NF- κ B activation was determined in lung tissue sections by immunohistochemistry, using an antibody directed against the p65 subunit (Santa Cruz Biotechnology, Santa Cruz, CA) and immunofluorescent detection. *See the online supplement for additional details regarding the NF- κ B activity assays.*

Lung Mechanics

Lung compliance and pressure–volume curves were measured as previously outlined (12). For details, *see the online supplement.*

Data Analysis

Data are expressed as means $\pm$ SEM. Significant differences between groups were identified by Student *t* test and analysis of variance.

RESULTS

Inhaled ENO Augments Lung SNO Levels

To determine whether inhaled ENO treatment results in augmentation of mouse lung SNO, we quantified SNO in BALF obtained from mice treated overnight (an approximately 15-h exposure time) with ENO. BALF SNO was increased greater than fourfold from baseline in mice that were exposed to 10 ppm ENO, with concentration-dependent increases between 10 and 100 ppm. (Figure 1). Higher BALF SNO levels persisted in the ENO-treated (10 ppm) mice after a 2.5-hour exposure to nebulized saline (Figure 2A). S-Nitrosylated protein appeared to be the primary component of BALF SNO as there was no detectable low molecular mass (<10 kD) SNO in the BALF in any treatment group.

A concomitant increase in mouse lung tissue (MLT) SNO was detected after ENO treatment as well (Figure 2B), suggesting that airway fluid and lung cell SNO are in equilibrium. As another method for quantifying lung tissue SNO, and as a direct measure of protein S-nitrosylation, we quantified S-nitrosylated NF- κ B p65 (SNO-p65) in MLT. NF- κ B p65 is known to be targeted by SNO for regulatory S-nitrosylation in respiratory epithelium and macrophages (17). Similar to the total SNO measurements in the BALF and MLT, ENO treatment significantly increased lung SNO-p65 levels (Figures 2C and 2D).

We found BALF SNO to be decreased in mice after aerosolized LPS exposure (Figure 2A), indicative of a cytokine-

Figure 3. 3-Nitrotyrosine (3-NT) immunoreactivity in representative lung sections from mice exposed to aerosolized lipopolysaccharide or saline (control) $\pm$ inhaled ethyl nitrite (ENO, 10 ppm). Insets depict detail of alveoli. Positive (sodium nitrite-treated) and negative (nonspecific antibody) controls are shown for comparison.

Figure 4. Effect of inhaled ethyl nitrite (ENO, 10 ppm) on lipopolysaccharide (LPS)-induced lung inflammation. (A) Total white blood cells (WBC) in bronchoalveolar lavage fluid. $n = 5$ per group; mean $\pm$ SEM; * $P < 0.005$ versus saline, ** $P < 0.01$ versus LPS. (B) Airway neutrophil and macrophage counts in bronchoalveolar lavage fluid. $n = 5$ per group; mean $\pm$ SEM; * $P < 0.01$ versus LPS alone. (C) Representative images of hematoxylin and eosin-stained lung sections from each treatment group, showing the LPS-induced influx of inflammatory cells into the airway.

induced perturbation in airway SNO homeostasis. The fact that both MLT SNO and lung SNO-p65 levels were also decreased after LPS exposure (Figures 2B and 2C) further supports a denitrosylating effect. On the other hand, ENO treatment (10 ppm) before the aerosolized LPS exposure augmented BALF SNO and MLT SNO-p65 levels to approximate those found in the saline controls.

As inhaled NO treatment is associated with increased lung protein nitration (3), we examined the lungs and airway fluid for 3-NT formation. 3-NT protein levels (as assessed by ELISA) were undetectable in the BALF from any of the treatment groups (saline, saline + ENO, LPS, LPS + ENO). 3-NT immunoreactivity in the airway epithelium was slightly enhanced after ENO treatment (10 ppm) (Figure 3) but was most

Figure 5. Effect of inhaled ethyl nitrite (ENO, 10 ppm) on lipopolysaccharide (LPS)-induced lung injury. (A) Static compliance; $n = 5$ per group; mean $\pm$ SEM; * $P < 0.01$ versus saline control ** $P < 0.01$ versus LPS alone. (B) Pressure-volume loops; $n = 3$ per group; mean $\pm$ SEM.

pronounced after LPS exposure, which is consistent with prior observations (19). However, ENO treatment before the LPS exposure resulted in marked attenuation of lung 3-NT immunoreactivity, indicating that peroxynitrite formation was, in fact, decreased in these mice.

ENO Attenuates LPS-induced Lung Inflammation and Injury

To examine the effects of ENO treatment on LPS-induced lung inflammation, we quantified inflammatory cells in the recovered BALF. Inhaled ENO or saline treatment alone did not alter resident airway inflammatory cell type or number in mice (Figure 4A). Aerosolized LPS exposure induced a marked recruitment of neutrophils into mouse lung airways (Figure 4B) as previously described (15). Prior treatment with inhaled ENO significantly decreased the influx of neutrophils into the airway of LPS-exposed mice. Histological examination of mouse lung sections revealed that the LPS-induced inflammation was most prominent in the distal airways and alveoli (Figure 4C). ENO treatment before the LPS exposure resulted in decreased inflammatory cell influx into these regions with diminished red blood cell extravasation and interstitial edema as well.

In addition to eliciting an inflammatory response, airway LPS administration is known to induce acute lung injury (20). As physiological measures of lung injury in our model, quasi-static compliance and pressure-volume (P-V) curves were generated in the saline- and LPS-treated mice with and without inhaled ENO treatment. One hour after completion of the aerosolized LPS exposure, lung compliance was dramatically decreased and P-V curves were flattened compared with those observed in saline controls (Figures 5A and 5B). Whereas ENO had no effect on lung mechanics in the saline control group,

ENO pretreatment before LPS exposure markedly improved compliance and the P-V curve contour, consistent with a decrease in lung injury.

ENO Inhibits NF- κ B Activation and Cytokine Expression in the Lung Airway

To determine whether ENO enhancement of airway SNO and lung SNO-p65 levels inhibits NF- κ B activation in the LPS-exposed mouse lung, we quantified NF- κ B DNA binding in nuclear lysates prepared from lung homogenate. LPS exposure alone induced an approximately fourfold increase in NF- κ B activation in the lung (Figure 6A). However, inhaled ENO treatment administered before LPS exposure resulted in significant diminution in lung NF- κ B activity.

To elucidate which cell population(s) in the lung accounted for the ENO-induced inactivation of NF- κ B, immunofluorescent staining of NF- κ B p65 was performed on fixed lung sections. NF- κ B was found to be activated by aerosolized LPS both in the bronchial and alveolar epithelium (Figures 6B and 6C; and see Figure E3 in the online supplement), consistent with prior observations (9). NF- κ B activation was most pronounced in the distal airways, similar to the histological foci of inflammation. Pretreatment with inhaled ENO markedly decreased NF- κ B p65 nuclear translocation in the bronchial and alveolar epithelium (Figure 6B), suggesting that the respiratory epithelium is the principal cell type affected by ENO inhibition of NF- κ B.

To ascertain whether inhaled ENO treatment suppresses the NF- κ B-dependent expression of inflammatory mediators in the lung, we quantified cytokine/chemokine levels in the BALF. ENO pretreatment significantly ameliorated the LPS-induced increase in KC, TNF- α , and IL-6 levels in the mouse airway (Figure 7). In addition, ENO pretreatment augmented the expression of the antiinflammatory cytokine IL-10, which is known to be negatively regulated by NF- κ B activation (21).

DISCUSSION

We found that LPS exposure in adult mice led to a decline in lung SNO that is associated with the onset of inflammation, and that pretreatment with inhaled ENO increases SNO in a dose-dependent manner, resulting in the attenuation of inflammation and subsequent lung injury. These findings are consistent with observations of SNO depletion in inflammatory airway disease (7, 8) and provide evidence that SNO metabolism plays a key role in the regulation of pulmonary immunity. In addition, our findings suggest that S-nitrosylation of NF- κ B (p50-p65) is a mechanism whereby inhaled ENO, secondary to airway SNO augmentation, attenuates LPS-induced lung inflammation.

Airway SNO levels are known to be deficient in asthma and cystic fibrosis (7, 8). Furthermore, airway activity of S-nitrosogluthathione reductase (GSNOR), an enzyme that metabolizes SNO, is increased in a mouse model of allergic asthma, with genetic deletion of GSNOR resulting in higher lung SNO levels and decreased airway hyperreactivity (22). At the cellular level, cytokine stimulation has been demonstrated to acutely decrease intracellular SNO leading to denitrosylation and activation of immune response proteins (23, 24). However, with prolonged cell stimulation, inducible nitric oxide synthase (NOS2) expression is induced, protein S-nitrosylation is increased, and immune response pathways are subsequently deactivated (17, 25). Taken together, these findings delineate SNO homeostasis as an important factor in regulating pulmonary inflammatory responses.

There are a number of potential mechanisms by which LPS exposure might induce a decrease in lung SNO. LPS increases

Figure 6. Effect of inhaled ethyl nitrite (ENO, 10 ppm) on lipopolysaccharide (LPS)-induced lung NF-κB activity. (A) Nuclear NF-κB p65 levels in lung homogenate. $n = 5$ per group; mean = SEM; * $P < 0.01$ LPS versus saline, LPS + ENO versus LPS alone. (B and C) Immunofluorescence localization of NF-κB p65 in representative sections from each treatment group: (B) bronchi; (C) alveoli. Red fluorophore, p65; blue (4',6-diamidino-2-phenylindole), nucleus. Differential interference contrast, low magnification, and Z-stack images are shown in Figure E3 in the online supplement.

a decrease in NO bioavailability and increased oxidation of thiol (3). Interestingly, we observed that inhaled ENO diminishes the LPS-induced increase in epithelial 3-NT formation (Figure 3), suggesting that enhancement of lung SNO levels inhibits airway superoxide accumulation, thereby attenuating peroxynitrite formation. In this regard, S-nitrosylation has been shown to inhibit NADPH oxidase and limit superoxide production (27). We also found significant red blood cell extravasation into the mouse alveoli in response to aerosolized LPS exposure (Figure 4C), and it is conceivable that reactions with extravasated hemoglobin may lower airway SNO (28). However, the fact that cytokine activation induces denitrosylating reactions in cultured respiratory epithelial cells (17) would argue against this explanation. We also considered that an LPS-induced perturbation of lung cell GSNOR and NOS2 activity, which regulate airway SNO levels (22), might function to lower airway SNO in this model. However, lung GSNOR activity is unchanged after LPS exposure and no significant alteration in NOS2 expression is observed in the lung until 6 hours after LPS exposure (see Figure E1 in the online supplement), making these enzymes unlikely to be responsible for the observed airway SNO depletion. Another possible mediator of airway SNO depletion in our model is thioredoxin, which has been shown to regulate protein denitrosylation (29). Although airway thioredoxin was not quantified in this study, airway fluid thioredoxin levels are known to be increased in patients with ALI (30).

A number of different pulmonary immune response pathways have been shown to be regulated by SNO. For example, S-nitrosylation modulates the activity of cyclooxygenase-2 (COX-2), c-Jun NH₂-terminal kinase-1 (JNK1), and surfactant protein D (SP-D), all of which mediate lung inflammation (31–33). However, in the present study, we found the mechanism by which ENO limits LPS-induced lung inflammation to be most consistent with SNO inhibition of NF-κB. ENO treatment markedly increased S-nitrosylation of NF-κB p50–p65 in the lung with a subsequent decrease in NF-κB activity on LPS exposure. We found ENO inhibition of NF-κB to be most prominent in the airway epithelium, where NF-κB activation is known to be essential in initiating the LPS inflammatory cascade and the subsequent development of lung injury (9). We also determined that LPS exposure robustly increases TNF-α, KC, and IL-6 levels in the BALF, with ENO treatment attenuating this response. All these inflammatory mediators are expressed by the respiratory epithelium and are NF-κB dependent (34, 35), thus providing further evidence that NF-κB inhibition is the mechanism by which ENO attenuates airway inflammation. Lung inflammation is partially resolved by leukocyte apoptosis and, as SNO inhibition of NF-κB induces apoptosis (25), we examined whether ENO treatment increases apoptosis of airway inflammatory cells. However, we found apoptosis not to be increased in either airway macrophages or neutrophils after ENO treatment, suggesting that enhancement of leukocyte apoptosis is not a mechanism by which lung inflammation is diminished in this model (see Figure E2 in the online supplement).

lung $O_2^{\cdot-}/H_2O_2$ production both intracellularly and in the airway fluid in response to immune activation of the epithelium and neutrophil influx, respectively (26). Under these conditions, SNO formation would be expected to be lower, because of

Figure 7. Effect of inhaled ethyl nitrite (ENO, 10 ppm) on bronchoalveolar lavage fluid cytokine/chemokine levels. Open columns = -ENO; solid columns = +ENO; n = 5 per group; mean + SEM; *P < 0.01 versus lipopolysaccharide alone.

The NF- κ B signaling cascade contains a number of proteins whose activity is regulated by SNO via S-nitrosylation. We have previously shown that in respiratory epithelium and macrophages, cytokine-induced NF- κ B activity is inhibited by S-nitrosylation of the p50-p65 heterodimer within the nucleus, resulting in decreased NF- κ B DNA binding (36). SNO also inhibits NF- κ B activation at the cytoplasmic level by inducing S-nitrosylation of I κ B kinase β (IKK2), an enzyme that regulates phosphorylation and degradation of the inhibitory NF- κ B protein I κ B α (24). Another cytoplasmic protein, MyD88, located upstream from the IKK complex in the Toll-like receptor-4 pathway, has also been shown to be inhibited by S-nitrosylation (37), resulting in a decrease in LPS-induced NF- κ B activation. Although we did not specifically evaluate which step in the NF- κ B pathway is inhibited by ENO, the fact that p65 does not undergo nuclear translocation after ENO treatment (Figures 6B and 6C) indicates that the regulatory target resides in the cytoplasm.

Although conventional inhaled NO therapy improves oxygenation in ALI/ARDS, its use has failed to demonstrate a mortality benefit in clinical trials (38, 39). This paradox may be explained, in part, by the chemical nature of NO, which reacts with molecular oxygen and superoxide to form toxic RNS that can exacerbate lung damage (2). In addition, NO might indiscriminately inactivate critical metalloproteins (e.g., cytochrome c oxidase), also inducing further lung injury (2). The reactions of ENO, unlike those of NO, are nitrosonium (NO⁺) based, which favors the formation of antiinflammatory SNO compounds and limits the potential for toxic NO reactions (11). Indeed, one study comparing inhaled ENO with NO as preventive therapy in a rat model of bronchopulmonary dysplasia found ENO to be superior in protection from hyperoxia-impaired postnatal lung development (12). Although ENO has not been clinically studied in ALI/ARDS, it has safely been administered to adults and neonates with pulmonary hypertension (13, 14). In summary, our results indicate that airway SNO augmentation by inhalation treatment with ENO is a potential therapeutic intervention for the prevention of ALI/ARDS and other inflammatory lung diseases in which airway SNO depletion is manifest.

Conflict of Interest Statement: H.E.M. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. E.N.P. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. Z.T.K. does not have a financial relationship with a commercial entity that has an interest in the subject of this manuscript. J.S.S. has a financial interest in N30 Pharmaceuticals, an early-stage biotech company developing S-nitrosothiols for respiratory use. He has previously received consultancy monies from Nitrox LLC (former name of N30) in amounts greater than \$10,000 annually. W.M.F. does not have a financial relationship

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