Nitric oxide synthase 3 contributes to ventilator-induced lung injury

Katerina Vaporidi, Roland C. Francis, Kenneth D. Bloch, and Warren M. Zapol

Anesthesia Center for Critical Care Research, Department of Anesthesia and Critical Care, Massachusetts General Hospital and Harvard Medical School, Boston Massachusetts

Submitted 25 September 2009; accepted in final form 6 May 2010

Vaporidi K, Francis RC, Bloch KD, Zapol WM. Nitric oxide synthase 3 contributes to ventilator-induced lung injury. Am J Physiol Lung Cell Mol Physiol 299:L150–L159, 2010. First published May 7, 2010; doi:10.1152/ajplung.00341.2009.—Nitric oxide synthase (NOS) deactivation or inhibition reduces ventilator-induced lung injury (VILI), but the responsible mechanisms remain incompletely defined. The aim of this study was to elucidate the role of endothelial NOS, NOS3, in the pathogenesis of VILI in an in vivo mouse model. Wild-type and NOS3-deficient mice were ventilated with high-tidal volume (HVVT; 40 ml/kg) for 4 h, with and without adding NO to the inhaled gas. Additional wild-type mice were pretreated with tetrahydrobiopterin and ascorbic acid, agents that can prevent NOS-generated superoxide production. Arterial blood gas tensions, histology, and lung mechanics were measured after 4 h of HVVT ventilation. The concentration of protein, IgM, cytokines, malondialdehyde, and 8-isoprostane were measured in bronchoalveolar lavage fluid (BALF). Myeloperoxidase activity, total and oxidized glutathione levels, and NOS-derived superoxide production were measured in lung tissue homogenates. HVVT ventilation induced VILI in wild-type mice, as reflected by decreased lung compliance, increased concentrations of protein and cytokines in BALF, and oxidative stress. All indices of VILI were ameliorated in NOS3-deficient mice. Augmenting pulmonary NO levels by breathing BH4 and ascorbic acid (22, 27). It is known that ROS can disrupt the endothelial barrier and induce inflammation (8, 9, 16, 40). The role of ROS in the pathogenesis of VILI is generally accepted (8, 29), but the sources of ROS have not been fully defined.

In the present study, we hypothesized that NOS3 contributes to the pathogenesis of VILI, not by producing NO, but rather by generating superoxide. To test this hypothesis, we compared the effect of HVVT ventilation in wild-type (WT) and NOS3−/− mice. We replenished NO in NOS3−/− mice by adding NO to the inhaled gas during mechanical ventilation. We evaluated oxidative stress and NOS-derived superoxide production in lungs of WT and NOS3−/− mice subjected to HVVT ventilation. Finally, we examined the effect of BH4 and ascorbic acid, which can prevent NOS3 uncoupling, in WT mice subjected to HVVT ventilation.

METHODS

Mouse model of VILI. Male C57BL/6 mice (WT) and NOS3−/− mice at 7–8 wk of age were studied as described in detail in the online data supplement (Supplemental data for this article is available at the AJP-Lung web site). The study was approved by the Subcommittee for Research Animal Care of the Massachusetts General Hospital. Briefly, after induction of anesthesia, a tracheotomy was performed, and mice were connected to a ventilator (Inspir; Harvard Apparatus, Boston, MA). Mice were ventilated on volume control mode, initially at a tidal volume (VT) of 8 ml/kg and respiratory rate (RR) 125 breaths/min for 1 h, to permit hemodynamic stabilization. The carotid artery was catheterized for blood pressure monitoring, fluid and anesthetic administration, and blood sampling. After 1 h, VT was increased to 40 ml/kg, and RR decreased to 60 breaths/min, at a PEEP of 1 cmH2O and FiO2 of 0.5 (maintained constant throughout the study). After 4 h of HVVT ventilation, blood was collected, and an inspiratory pressure-volume curve of the respiratory system was obtained by slow inflation of the lungs, as previously described (34). Subsequently, bronchoalveolar lavage was performed, and the lungs were collected for further analysis. As controls, additional animals of both genotypes were anesthetized and ventilated briefly (<1 min) until paralyzed, with a VT of 8 ml/kg and RR of 125 breaths/min, whereupon an inspiratory pressure volume curve was obtained, followed by bronchoalveolar lavage and tissue collection. For histological evaluation, lungs from mice not subjected to BALF collection were inflated with 4% paraformaldehyde at a transpulmonary pressure of 25 cmH2O. A detailed description of the experimental procedure is provided in the online data supplement and in Supplementary Fig. S1.
The tidal volume and the duration of ventilation were chosen based on results from pilot experiments, which revealed that these conditions caused VILI in WT mice with a reproducible alteration in lung mechanics and inflammation.

Experimental groups. We studied nine groups of mice. We studied WT and NOS3<sup>-/-</sup> mice under control conditions and after 4 h of HVT ventilation. In addition, we studied WT and NOS3<sup>-/-</sup> mice subjected to HVT ventilation while receiving inhaled NO, at either a concentration of 5 or 50 parts per million (ppm; Medical-Technical Gases, Medford, MA) added to inspiratory gas from the initiation of mechanical ventilation. Finally, we studied a group of WT mice that was treated with BH<sub>4</sub> (Schricks Laboratory, Switzerland) dissolved in an ascorbic acid solution (Bioniche Pharma, Lake Forest, IL; 100 mg/kg of both BH<sub>4</sub> and ascorbic acid, injected ip) before the initiation of HVT ventilation. The number of animals in each experimental group is presented in Table 1. The number of animals studied in each group was based on our pilot studies that revealed at least five mice in each group were required to show a difference in VILI between WT and NOS3<sup>-/-</sup> mice subjected to HVT ventilation. Additional mice were included to provide adequate samples for biochemical and histological studies and to confirm the stability of the model over time.

Evaluation of lung injury and inflammation. Protein concentration in BALF was measured with a bicinchoninic acid assay (Pierce Chemical, Rockford, IL). IgM levels in BALF were measured with an ELISA (Genway Biotech). The levels of the proinflammatory cytokines, IL-6, TNF<sub>a</sub>, and macrophage inflammatory protein-2 (MIP-2), were measured in BALF and plasma using ELISAs (R&D Systems, Minneapolis, MN). Total and differential cell counts in BALF and lung myeloperoxidase (MPO) activity were performed as described in the online data supplement. Because sample quantities from individual animals were insufficient to perform all assays, a randomly chosen subset of samples was used for each assay. The number of samples used in each assay is reported in the figure legends.

Paraffin-embedded lung sections were sectioned 6 µm thick and stained with hematoxylin and eosin for histology. Staining for neutrophils was performed in the same lung sections using antimouse neutrophil monoclonal antibody (CL8993 AP; Cedarline Laboratories, Ontario, Canada).

Measurement of nitric oxide metabolites and NOS synthase gene expression. Total nitrite/nitrate levels in BALF were measured using a fluorimetric assay (Cayman Chemical). Methods for measurement of pulmonary NOS1, NOS2, and NOS3 mRNA levels are described in the online data supplement.

Evaluation of oxidative stress and ROS production. Malondialdehyde (MDA) levels in BALF were measured using a spectrophotometric assay for MDA (Oxis International, Foster City, CA). BALF 8-isoprostanate levels were measured using an EIA assay for 8-isoprostane (Cayman Chemical). Total and oxidized glutathione levels in lung tissue were measured using an enzymatic assay (Cayman Chemical).

Superoxide production was measured in lung homogenates using a lucigenin-enhanced chemiluminescence assay supplemented with β-nicotinamide adenine dinucleotide 2’-phosphate reduced tetrasodium salt (NADPH) in the presence and absence of nitro-l-arginine methyl ester (l-NAME), a NOS inhibitor, as described in the online data supplement.

Statistical analysis. Data were compared by two-way ANOVA (the non-parametric tests Mann-Whitney rank sum test and Kruskal-Wallis were used when normality and equal variance test failed). When the P value was less than 0.05, the Bonferroni post hoc test was applied. All analyses were performed using SigmaStat statistical software. All data in text and tables are expressed as means ± SD, and data are presented in figures as box plots. Significance was defined as P < 0.05.

RESULTS

Effects of HVT ventilation on WT and NOS3<sup>-/-</sup> mice. In all mice, peak inspiratory pressure (PIP) at the initiation of HVT (40 ml/kg) was 36 ± 0.5 cmH<sub>2</sub>O. Shortly after the initiation of HVT ventilation, PIP decreased modestly in all mice, possibly due to resolution of atelectasis. In WT mice, PIP increased after 2 h of HVT ventilation and was 38.8 ± 2.2 cmH<sub>2</sub>O after 4 h (P < 0.001 vs. PIP at initiation of HVT; Fig. 1A). In NOS3<sup>-/-</sup> mice, PIP was not different at the initiation and at the end of 4 h of HVT ventilation.

The pressure-volume curve of the respiratory system was not different in control WT and NOS3<sup>-/-</sup> mice. HVT ventilation for 4 h decreased lung compliance in WT and NOS3<sup>-/-</sup> mice, as reflected by the downward shift of the pressure-volume curve of the respiratory system (Fig. 1B). However, after 4 h of HVT ventilation, lung compliance was higher in NOS3<sup>-/-</sup> mice than in WT mice. In WT mice, the development of pulmonary edema, as indicated by the decline in lung compliance, was associated with impaired oxygenation, as reflected by a decreased PaO<sub>2</sub> and an increased alveolar-arterial oxygen difference [D(A-a)O<sub>2</sub>, Table 2]. After HVT ventilation, D(A-a)O<sub>2</sub> was less in NOS3<sup>-/-</sup> mice than in WT mice (34 ± 13 vs. 137 ± 91 mmHg, P < 0.001). There were no differences in PaCO<sub>2</sub>, pH, and HCO<sub>3</sub> between the two genotypes after 4 h of HVT ventilation (Table 2).

Concentrations of protein and IgM in BALF did not differ in control WT and NOS3<sup>-/-</sup> mice. BALF protein concentrations were greater in WT and NOS3<sup>-/-</sup> mice ventilated with HVT than in corresponding control mice consistent with increased alveolar-capillary permeability (Fig. 2). Moreover, IgM, which, due to its high-molecular-weight (900 kDa), is largely excluded from the BALF under normal conditions (19), was increased in the BALF of WT and NOS3<sup>-/-</sup> mice exposed to HVT ventilation (Fig. 3). However, BALF total protein and IgM concentrations were lower in NOS3<sup>-/-</sup> mice exposed to HVT ventilation than in similarly ventilated WT mice (Figs. 2 and 3).

Concentrations of proinflammatory cytokines in BALF and plasma, as well as leukocyte numbers in BALF and lung tissue, did not differ between control WT and NOS3<sup>-/-</sup> mice. HVT ventilation induced an inflammatory response in the lungs of WT and NOS3<sup>-/-</sup> mice, as indicated by increased levels of IL-6, TNF<sub>a</sub>, and MIP-2 in BALF (Figs. 4 and 5). HVT ventilation was also associated with increased plasma IL-6 levels in both genotypes (Fig. 5C). TNF<sub>a</sub> and MIP-2 were not detected in the plasma of WT or NOS3<sup>-/-</sup> mice after HVT ventilation. BALF concentrations of all measured proinflammatory cytokines were less in NOS3<sup>-/-</sup> mice exposed to HVT.

Table 1. Experimental groups and number of animals in each group

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Control</th>
<th>HVT&lt;sub&gt;1&lt;/sub&gt;</th>
<th>HVT&lt;sub&gt;2&lt;/sub&gt;</th>
<th>HVT&lt;sub&gt;3&lt;/sub&gt;</th>
<th>HVT&lt;sub&gt;4&lt;/sub&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Genotype</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>WT</td>
<td>11</td>
<td>19</td>
<td>10</td>
<td>7</td>
<td>6</td>
</tr>
<tr>
<td>NOS3&lt;sup&gt;-/-&lt;/sup&gt;</td>
<td>11</td>
<td>19</td>
<td>19</td>
<td>10</td>
<td>7</td>
</tr>
</tbody>
</table>

Number of animals in each of 9 study groups: control group; WT mice subjected to HVT ventilation (VT = 40 ml/kg) without (HVT<sub>1</sub>) or with 5 ppm (HVT<sub>2</sub>) or 50 ppm (HVT<sub>3</sub>) inhaled NO; and WT mice treated with BH<sub>4</sub> and ascorbic acid (HVT<sub>4</sub>).
ventilation than in similarly ventilated WT mice. Moreover, plasma IL-6 concentrations were less in NOS3+/− mice than in WT mice after HVT ventilation.

HVT ventilation induced neutrophil infiltration into the lungs in both genotypes, as indicated by increased lung tissue MPO activity (Fig. 6) and BALF neutrophil counts (data not shown). Lung tissue MPO activity was less in NOS3+/− than in WT mice exposed to HVT ventilation. The increase in BALF neutrophils induced by HVT ventilation was similar in the two genotypes.

The histological appearance of lungs from control WT and NOS3+/− mice was not different (Fig. 7A). HVT ventilation induced inflammatory cell infiltration and thickening of alveolar walls of WT mice. HVT ventilation caused less inflammatory cell infiltration and alveolar wall thickening in NOS3+/− than in WT mice. Immunohistochemistry revealed a marked increase in the number of neutrophils in the lungs of all mice exposed to HVT ventilation (Fig. 7, B and C). In NOS3+/− mice exposed to HVT ventilation, lung neutrophil infiltration was less than that in similarly ventilated WT mice.

In control WT and NOS3+/− mice, the levels of NO metabolites and nitrite/nitrate in BALF were not different (Fig. 8). HVT ventilation increased BALF NO metabolite concentrations twofold in WT mice but did not alter levels in NOS3+/− mice. The levels of NOS1 and NOS2 mRNA in the lungs were not different between genotypes in control mice and were unaffected by HVT ventilation (data not shown).

**Effect of inhaled NO on the development of VILI in NOS3+/− and WT mice.** To test the hypothesis that NOS3-derived NO contributes to the development of VILI, we subjected NOS3+/− mice to the same HVT ventilation protocol and replenished NO in the lungs by adding a low concentration (5 ppm) of NO gas to the ventilation gas (beginning from the initiation of mechanical ventilation). We found that breathing 5 ppm NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. Lung compliance and oxygenation after 4 h of HVT ventilation were not different in NOS3+/− mice ventilated with and without inhaled NO (Fig. 1B and Table 2). Total protein and IL-6 concentrations in BALF were similar in NOS3+/− mice ventilated without or with 5 ppm NO, as were lung MPO activity and leukocyte accumulation (Figs. 3, 5, 7, and 8).

To ascertain if a higher concentration of inhaled NO would induce injury in NOS3+/− mice, we tested the effect of inhaling 50 ppm NO in mice subjected to the same HVT ventilation protocol. We found that, even at higher concentrations, inhaled NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. No differences were observed in lung compliance, oxygenation, BALF protein, or IL-6 concentrations, as well as reduced pulmonary neutrophil infiltration and thickening of alveolar walls of WT mice. HVT ventilation caused less inflammatory cell infiltration and thickening of alveolar walls in NOS3+/− mice than in similarly ventilated WT mice.

**Effect of inhaled NO on the development of VILI in NOS3+/− and WT mice.** To test the hypothesis that NOS3-derived NO contributes to the development of VILI, we subjected NOS3+/− mice to the same HVT ventilation protocol and replenished NO in the lungs by adding a low concentration (5 ppm) of NO gas to the ventilation gas (beginning from the initiation of mechanical ventilation). We found that breathing 5 ppm NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. Lung compliance and oxygenation after 4 h of HVT ventilation were not different in NOS3+/− mice ventilated with and without inhaled NO (Fig. 1B and Table 2). Total protein and IL-6 concentrations in BALF were similar in NOS3+/− mice ventilated without or with 5 ppm NO, as were lung MPO activity and leukocyte accumulation (Figs. 3, 5, 7, and 8).

To ascertain if a higher concentration of inhaled NO would induce injury in NOS3+/− mice, we tested the effect of inhaling 50 ppm NO in mice subjected to the same HVT ventilation protocol. We found that, even at higher concentrations, inhaled NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. No differences were observed in lung compliance, oxygenation, BALF protein, or IL-6 concentrations, as well as reduced pulmonary neutrophil infiltration and thickening of alveolar walls of WT mice. HVT ventilation caused less inflammatory cell infiltration and thickening of alveolar walls in NOS3+/− mice than in similarly ventilated WT mice.

**Effect of inhaled NO on the development of VILI in NOS3+/− and WT mice.** To test the hypothesis that NOS3-derived NO contributes to the development of VILI, we subjected NOS3+/− mice to the same HVT ventilation protocol and replenished NO in the lungs by adding a low concentration (5 ppm) of NO gas to the ventilation gas (beginning from the initiation of mechanical ventilation). We found that breathing 5 ppm NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. Lung compliance and oxygenation after 4 h of HVT ventilation were not different in NOS3+/− mice ventilated with and without inhaled NO (Fig. 1B and Table 2). Total protein and IL-6 concentrations in BALF were similar in NOS3+/− mice ventilated without or with 5 ppm NO, as were lung MPO activity and leukocyte accumulation (Figs. 3, 5, 7, and 8).

To ascertain if a higher concentration of inhaled NO would induce injury in NOS3+/− mice, we tested the effect of inhaling 50 ppm NO in mice subjected to the same HVT ventilation protocol. We found that, even at higher concentrations, inhaled NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. No differences were observed in lung compliance, oxygenation, BALF protein, or IL-6 concentrations, as well as reduced pulmonary neutrophil infiltration and thickening of alveolar walls of WT mice. HVT ventilation caused less inflammatory cell infiltration and thickening of alveolar walls in NOS3+/− mice than in similarly ventilated WT mice.

**Effect of inhaled NO on the development of VILI in NOS3+/− and WT mice.** To test the hypothesis that NOS3-derived NO contributes to the development of VILI, we subjected NOS3+/− mice to the same HVT ventilation protocol and replenished NO in the lungs by adding a low concentration (5 ppm) of NO gas to the ventilation gas (beginning from the initiation of mechanical ventilation). We found that breathing 5 ppm NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. Lung compliance and oxygenation after 4 h of HVT ventilation were not different in NOS3+/− mice ventilated with and without inhaled NO (Fig. 1B and Table 2). Total protein and IL-6 concentrations in BALF were similar in NOS3+/− mice ventilated without or with 5 ppm NO, as were lung MPO activity and leukocyte accumulation (Figs. 3, 5, 7, and 8).

To ascertain if a higher concentration of inhaled NO would induce injury in NOS3+/− mice, we tested the effect of inhaling 50 ppm NO in mice subjected to the same HVT ventilation protocol. We found that, even at higher concentrations, inhaled NO did not increase lung injury in NOS3+/− mice exposed to HVT ventilation. No differences were observed in lung compliance, oxygenation, BALF protein, or IL-6 concentrations, as well as reduced pulmonary neutrophil infiltration and thickening of alveolar walls of WT mice. HVT ventilation caused less inflammatory cell infiltration and thickening of alveolar walls in NOS3+/− mice than in similarly ventilated WT mice.
Oxidative stress and sources of superoxide in WT and NOS3–/– mice subjected to HV T ventilation. The observation that inhaled NO did not induce injury in NOS3–/– mice, and even ameliorated VILI in WT mice at low concentrations, indicated that NOS3 does not contribute to VILI via NO production. It is known that NOS3 can become uncoupled and produce superoxide instead of NO (13) and that oxidative stress contributes to VILI (8, 29). Oxidative stress in mice subjected to HV T ventilation was evaluated by measuring the levels of lipid oxidation products, MDA and 8-isoprostanate and the ratio of total to oxidized glutathione levels. HV T ventilation increased MDA levels in BALF (Fig. 9A), as well as lung and kidney tissues (Supplementary Fig. S3), in WT mice, but not in NOS3–/– mice. HV T ventilation increased 8-isoprostanate levels in BALF from both WT and NOS3–/– mice, but levels were greater in WT mice (Fig. 9B). The ratio of total to oxidized glutathione levels was reduced in lungs of WT mice subjected to HV T ventilation but not in those from similarly treated NOS3–/– mice (Fig. 9C). Together, these findings strongly suggest that presence of NOS3 contributes to the oxidative stress caused by HV T ventilation.

To investigate whether NOS3 itself contributes to the observed increase in lung oxidative stress caused by HV T ventilation, we measured superoxide production in lung homogenates, using lucigenin-enhanced chemiluminescence, in the presence and absence of a NOS inhibitor, L-NAME. We found that superoxide production was twofold greater in lungs of WT mice subjected to HV T ventilation than in lungs from control WT mice (319 ± 97 vs. 163 ± 89 RLU·s−1·mg−1 protein, respectively; P < 0.05). Superoxide production tended to be greater in the lungs of NOS3–/– mice subjected to HV T ventilation than in the lungs of control NOS3–/– mice (260 ± 85 vs. 154 ± 44 RLU·s−1·mg−1 protein, respectively; P = 0.06). Addition of superoxide scavengers, superoxide dismutase, and Tiron, to the reaction buffer abolished the chemiluminescence signal, confirming the specificity of the assay (data not shown). L-NAME did not alter superoxide production in the lungs of control WT and NOS3–/– mice or in NOS3–/– mice subjected to HV T ventilation (Fig. 9B). In contrast, L-NAME markedly inhibited superoxide production in the lungs of WT mice subjected to HV T ventilation. The finding that HV T ventilation induces NOS-inhibitable superoxide production in WT lungs, but not NOS3–/– lungs, strongly suggests that NOS3 itself contributes to the increased superoxide generation seen in WT mice.
Effect of treatment with BH4 and ascorbic acid on VILI in WT mice. Having observed that NOS3 can contribute to superoxide production by lungs of WT mice subjected to HV T ventilation, we sought to determine if BH4 and ascorbic acid, antioxidants that can prevent NOS3 uncoupling and superoxide production, would ameliorate VILI in WT mice. We found that after 4 h of HV T ventilation, lung compliance was greater in WT mice treated with BH4 and ascorbic acid than in untreated WT mice (Fig. 1B). Oxygenation was also better in BH4- and ascorbic acid-treated mice than in untreated WT mice [D(A-a)O2: 35 ± 26 vs. 137 ± 91 mmHg, P = 0.003; Table 2]. BALF protein concentrations were lower in BH4- and ascorbic acid-treated mice subjected to HV T ventilation than in untreated WT mice breathing 5 ppm NO than in WT mice not receiving NO (#P < 0.001). BALF IL-6 concentrations after HV T ventilation were greater in WT mice breathing 50 ppm NO than in WT mice not receiving NO (iP < 0.01). BALF IL-6 concentrations after HV T ventilation were greater in WT mice breathing 50 ppm NO than in WT mice not receiving NO (iP < 0.01). N = 3–4 per group as controls and 6–10 per group for HV T ventilation. Data are presented in box plots as in Fig. 2.

DISCUSSION

The main finding of this study is that NOS3 is an important participant in the pathogenesis of murine VILI in vivo. HV T induced a greater decrease in lung compliance and a more marked increase in lung permeability, inflammation, and oxidative stress in WT than in NOS3−/− mice. Replenishment of NO by adding NO to the inhaled gas did not increase lung injury in NOS3−/− mice exposed to HV T ventilation. HV T ventilation induced i-NAME-inhibitable superoxide production in WT but not in NOS3−/− mice. Finally, VILI was reduced in WT mice by pretreatment with the antioxidants BH4 and ascorbic acid that can prevent NOS3 uncoupling. Together, our data support the hypothesis that NOS3 contributes to VILI in a NO-independent manner, by increasing the production of superoxide likely by uncoupled NOS3.
In the present study, we used a mouse model in which HVT ventilation (40 ml/kg) consistently disrupted the alveolar-capillary barrier (resulting in high-permeability pulmonary edema) and induced inflammation, hallmarks of VILI (14, 20, 29, 42). We observed that HVT ventilation-induced pulmonary edema was greater in WT mice than in NOS3−/− mice, as indicated by the downward shift of the pressure-volume curve and impaired arterial oxygenation. Total protein and IgM concentrations in BALF were higher in WT than in NOS3−/− mice after HVT ventilation, consistent with more marked disruption of the alveolar-capillary barrier in WT mice. Moreover, the levels of proinflammatory cytokines in BALF and lung neutrophil infiltration were greater in WT than in NOS3−/− mice exposed to HVT ventilation. Our in vivo results demonstrate that NOS3 contributes to both HVT-induced pulmonary edema and lung inflammation.

HVT ventilation is associated with increased levels of NO metabolites in BALF (15, 17). In our study, BALF concentrations of NO metabolites were increased in WT but not in NOS3−/− mice subjected to HVT ventilation. Pulmonary NOS1 and NOS2 mRNA levels were not different in WT and NOS3−/− mice suggesting that the observed differences in BALF NO metabolite concentrations between the two genotypes were attributable to NOS3.

To determine whether reduced pulmonary NO levels were responsible for the protective effects of NOS3 deficiency, we examined the impact of replenishing pulmonary NO levels in NOS3−/− mice subjected to HVT ventilation. We studied the impact of a low dose of inhaled NO (5 ppm), similar to the concentration previously used to restore the WT phenotype in NOS3−/− mice (5), and a higher dose (50 ppm), similar to that previously used to restore the WT phenotype in NOS2−/− mice (39, 41). We found that addition of inhaled NO at either low or high concentrations had no effect on the response of NOS3−/− mice to HVT ventilation. These findings suggest that NO produced by NOS3 is insufficient to account for the greater VILI seen in WT mice than in NOS3−/− mice. We also examined the effect of breathing the same concentrations of NO in WT mice subjected to HVT ventilation. Interestingly, we found that breathing 5 ppm NO protected WT mice from the development of VILI. Our results are in agreement with previous studies showing that low concentrations of inhaled NO were protective in other models of lung injury (6, 18, 21, 32, 35). In contrast, we found that breathing higher concentrations of NO did not prevent lung injury in WT mice subjected to HVT ventilation. These results suggest that high lung NO levels have deleterious effects that can overwhelm the positive effects of low NO concentrations.

The contribution of NOS3 in VILI has been examined in other studies with conflicting results. Consistent with our study, Schmidt et al. (36), using isolated-perfused mouse lungs, found that NOS3 deficiency reduces HVT-induced pulmonary edema and suggested that NO and cyclic GMP signaling contribute to HVT-induced barrier disruption. In contrast, Peng et al. (31) recently reported that pulmonary edema was more marked in NOS3−/− than in WT mice. Takenaka et al. (38) observed that transgenic overexpression of NOS3, leading to increased pulmonary NO levels, protected mice from VILI. We found that increasing pulmonary NO levels with low concentrations of inhaled NO protected WT mice from VILI. It is of note that Peng and colleagues and Takenaka and colleagues both used a VT of 20 ml/kg, whereas we used a VT of 40 ml/kg. In our pilot studies, we found that ventilation with 40 ml/kg caused a greater and more consistent degree of lung injury and inflammation than did 20 ml/kg.

In our model, the deleterious effects of NOS3 in VILI did not appear to be mediated by NO, leading us to investigate whether another NOS3 product could contribute to HVT-induced lung injury. It is known that NOS can produce superoxide instead of NO when the levels of BH4 or L-arginine are insufficient (13), a process referred to as NOS uncoupling. Superoxide derived from uncoupled NOS3 contributes to vascular dysfunction both in animal models and in human beings with hypertension, hypercholesterolemia, and diabetes mellitus (13). HVT ventilation could induce NOS3 uncoupling by depleting BH4 and/or arginine. HVT ventilation may deplete arginine by inducing expression of arginases (2), but in our model, HVT did not increase lung arginase I and II gene expression (data not shown). Alternatively, ROS generated in the lungs during HVT ventilation by xanthine oxidase and neutrophil NADPH oxidase (1, 10, 33) may inactivate BH4 (22), thereby uncoupling NOS3 (23) and leading to additional superoxide production. In our study, HVT ventilation increased oxidative stress in WT mice, as indicated by increased MDA and 8-isoprostane levels in BALF, reduced total to oxidized glutathione levels in lung tissue, and increased MDA in both lungs and kidneys. NOS-inhibitable superoxide production in lungs from WT but not from NOS3−/− mice exposed to HVT ventilation suggests that NOS3 itself can contribute to lung superoxide production. Superoxide, generated by NOS and other enzymes, likely contributes to the development of VILI via several mechanisms. Superoxide can induce lipid peroxidation leading to cell membrane destruction (37) and increased vascular permeability (40). Superoxide can increase ROS production from neutrophils and macrophages and affect the

Fig. 6. Lung myeloperoxidase (MPO) activity from control WT and NOS3−/− mice and WT and NOS3−/− mice ventilated with HVT (40 ml/kg) for 4 h, with or without 5 ppm inhaled NO (INO ppm). Lung MPO activity was greater in WT and NOS3−/− mice subjected to HVT ventilation than in the corresponding controls (**P < 0.001 and #P < 0.01, respectively). Lung MPO activity after HVT ventilation was less in NOS3−/− mice and in WT mice breathing 5 ppm NO than in WT mice not receiving NO (†P < 0.01 and ‡P < 0.001, respectively). Data are expressed as MPO activity units per milligram of tissue; n = 3–4 per group for control and 6–9 per group for HVT ventilation. Data are presented in box plots as in Fig. 2.
activity of other ROS-producing enzymes (11, 28). By this means, NOS3 uncoupling can contribute to VILI by amplifying ROS production from other enzymes, such as NADPH oxidase and xanthine oxidase, and increase oxidative stress.

We found that treatment with BH4 and ascorbic acid, antioxidants that can prevent NOS3 uncoupling (22, 27), ameliorated VILI in WT mice. BH4 serves as an electron donor in NO synthesis and is indispensable for NOS3 function (1), whereas ascorbic acid recycles the oxidized BH3 radical to BH4 (22, 27). Although BH4 and ascorbic acid may protect against VILI via mechanisms other than NOS3 coupling, our findings support the hypothesis that NOS3-derived superoxide plays an important role in VILI.

Our study has several limitations. To evaluate the role of NOS3-derived NO in VILI, we replenished lung NO levels by adding NO gas to the gas mixture used to ventilate the mice we studied. Although the location and concentration of NO may differ in WT mice expressing NOS3 and in NOS3−/− mice breathing NO, we and others have successfully used inhaled NO to restore a WT phenotype in NOS-deficient mice (5, 39, 41). Moreover, use of inhaled NO permitted us to discriminate between the roles of NO and superoxide in vivo in a manner that was not achievable with NOS inhibitors, which block the synthesis of both NO and superoxide by NOS, or with NO-donor compounds, which can cause systemic hypotension. We found that NOS3 contributes to oxidative stress and superoxide production in lungs of WT mice subjected to HVT ventilation, which we attributed to NOS3 uncoupling. We did not find increased monomerization of NOS3 (data not shown), which has been associated with NOS3 uncoupling (27). However, other investigators have reported that purified NOS monomers are inactive (4, 7) and that the presence of NOS monomers is not required for NOS uncoupling (13). In contrast to the observations of Peng and colleagues (31), we also did not find increased levels of nitrotyrosine (data not shown), an index of peroxynitrite generation, in the lungs of mice ventilated with...
high tidal volumes. These differing results may be attributable
differences in the mouse models of VILI and/or the methods
used to measure lung nitrotyrosine levels.

The observation that HV ventilation is associated with
increased superoxide production by NOS3 provides a novel
therapeutic target for VILI. Pharmacological interventions
aimed at preventing NOS3 uncoupling, such as administration
of BH4 or 5-methyltetrahydrofolate (3, 27), that have shown
promising results of experimental studies in vascular disease,
may also play a role in the prevention and treatment of VILI.

In summary, our data suggest that NOS3 contributes to the
alveolar-capillary barrier dysfunction, pulmonary inflamma-

Fig. 8. BALF concentrations of NO metabolites (nitrite/nitrate, NOx) from
control WT and NOS3−/− mice and WT and NOS3−/− mice ventilated with
HV (40 ml/kg) for 4 h. BALF NOx levels were greater in WT mice subjected
to HV ventilation than in WT controls (*P < 0.001). BALF NOx levels after
HV ventilation were less in NOS3−/− mice than in WT mice (#P < 0.01).
N = 5–8 per group. Data are presented in box plots as in Fig. 2.

Fig. 9. A: BALF concentrations of malondialdehyde (MDA) from control WT
and NOS3−/− mice and WT and NOS3−/− mice ventilated with HV (40
ml/kg) for 4 h. BALF MDA levels were greater in WT mice subjected
to HV ventilation than in WT controls (*P < 0.01), but were unchanged in NOS3−/−
mice. BALF MDA levels after HV ventilation were less in NOS3−/− mice
than in WT mice (#P < 0.01). Data are presented in box plots as in Fig. 2; n =
6–8 per group. B: BALF concentrations of 8-isoprostane from control WT
and NOS3−/− mice and WT and NOS3−/− mice ventilated with HV (40 ml/kg)
for 4 h. BALF 8-isoprostane levels were greater in WT and NOS3−/− mice
subjected to HV ventilation than in corresponding controls (*P < 0.001 and
#P < 0.01, respectively). BALF MDA levels after HV ventilation were less
in NOS3−/− than WT mice (P < 0.01). Data are presented in box plots as in
Fig. 2; n = 5 per group. C: the ratio of total to oxidized glutathione levels
(GSH/GSSG) in lung tissues from control WT and NOS3−/− mice, and WT
and NOS3−/− mice ventilated with HV (40 ml/kg) for 4 h. GSH/GSSG was
less in WT mice subjected to HV ventilation than in WT controls (*P < 0.05).
HV ventilation did not reduce GSH/GSSG in NOS3−/− mice, and GSH/
GSSG was greater in NOS3−/− mice subjected to HV ventilation than in
similarly ventilated WT mice (*P < 0.05). Data are presented in box plots as in
Fig. 2; n = 5 per group. D: NOS-derived superoxide production in lung
homogenates from control WT and NOS3−/− mice and WT and NOS3−/− mice
ventilated with HV (40 ml/kg) for 4 h. NOS-derived superoxide production
was estimated from the difference in lucigenin-enhanced chemiluminescence
(LEC) measured in the absence and presence of L-NAME (1 mM).
L-NAME-inhibitable superoxide production in lung homogenates
is expressed as the difference in chemiluminescence (RLU) per second per
milligram of protein, n = 5 per group. Data are presented in box plots as in Fig. 2.
tion, and oxidative stress associated with VILI. NOS3-derived NO appears insufficient to cause VILI, as inhaled NO does not worsen VILI in NOS3−/− mice, and, in fact, at low concentrations, NO can ameliorate VILI in WT mice. These findings suggest that NOS3, and in particular superoxide generation by NOS3, may represent a novel therapeutic target for the prevention of VILI.

ACKNOWLEDGMENTS

We thank Drs. Yasuko Nagasaka, Patricio Leyton, and Emmanuel Buys and Michael J. Raher, Kristen Rauwerdink, Yuko Beppu, and Dr. Rong Liu for advice and skillful assistance; Dr. Rosemary Jones for advice interpreting lung histology; and Dr. Hui Zheng for assistance in statistical analysis.

GRANTS

This work was supported, in part, by National Heart, Lung, and Blood Institute Grant HL-42397 (to W. M. Zapol) and by a sponsored research agreement between the Massachusetts General Hospital (MGH) and IKARIA.

DISCLOSURES

The MGH has obtained patents relating to the use of inhaled nitric oxide and has licensed them to IKARIA and Linde Gas Therapeutics, Lidingo, Sweden. W. M. Zapol receives royalties and K. D. Bloch has received grants from IKARIA.

REFERENCES


