Original Paper

Biology of the Neonate

Biol Neonate 2000;78:198–206

High-Dose Inhaled Nitric Oxide and Hyperoxia Increases Lung Collagen Accumulation in Piglets

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Key Words

Pulmonary matrix degradation · Nitric oxide · Hyperoxia · Hydroxyproline · Matrix metalloproteinases

Abstract

Nitric oxide (NO), a pro-oxidant gas, is used with hyperoxia (O_2) to treat neonatal pulmonary hypertension and recently bronchopulmonary dysplasia, but great concerns remain regarding NO's potential toxicity. Based on reports that exposure to oxidant gases results in pulmonary extracellular matrix injury associated with elevated lavage fluid levels of extracellular matrix components, we hypothesized that inhaled NO with or without hyperoxia will have the same effect. We measured alveolar septal width, lung collagen content, lavage fluid hydroxyproline, hyaluronan and laminin levels in neonatal piglets after 5 days' exposure to room air (RA), RA + 50 ppm NO (RA + NO), O_2 ($FiO_2 > 0.96$) or O_2 + NO. Matrix metalloproteinase (MMP) activity and MMP-2 mRNA were also measured. In recovery experiments, we measured lung collagen content in piglets exposed to RA + NO or O_2 + NO and then allowed to recover for 3 days. The results show that lung collagen increased 4-fold in the RA + NO piglets, the O_2 and O_2 + NO groups had only a 2-fold elevation relative to RA controls. Unlike the RA + NO piglets, the O_2 and O_2 + NO groups had more than 20-fold elevation in lung lavage fluid hydroxyproline compared to the RA group. O_2 and O_2 + NO also had increased lung MMP activity, extravascular water, and lavage fluid proteins. MMP-2 mRNA levels were unchanged. After 3 days' recovery in room air, the RA + NO groups' lung collagen had declined from 4-fold to 2-fold above the RA group values. The O_2 + NO group did not decline. Alveolar septal width increased significantly only in the O_2 and O_2 + NO groups. We conclude that 5 days' exposure to NO does not result in pulmonary matrix degradation but instead significantly increases lung collagen content. This effect appears potentially reversible. In contrast, hyperoxia exposure with or without NO results in pulmonary matrix degradation and increased lung collagen content. The observation that NO increased lung collagen content represents a new finding and suggests NO could potentially induce pulmonary fibrosis.

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Introduction

Inhalational exposure to oxidant gases may result in pulmonary matrix injury [1–4]. In newborns, pulmonary injury may progress to development of bronchopulmonary dysplasia, the manifestations of which include persistent chronic hypoxemia, in part from elevated pulmonary vascular pressures. Nitric oxide (NO), a pro-oxidant gas which may also exhibit antioxidant properties under some conditions, is used in conjunction with hyperoxia to treat neonatal pulmonary hypertension and more recently has been reported to improve oxygenation in infants with bronchopulmonary dysplasia [5–9]. However, great concerns exist regarding NO's potential toxicity. In contrast to the reports about the effects of hyperoxia on the lung extracellular matrix, limited data exist on the effect of inhaled NO on the matrix. [1–4, 10–12]. NO toxicity is usually attributed to its oxidative capabilities, especially that of its metabolites, nitrogen dioxide (NO_2) and peroxynitrite [13–15]. In the presence of oxygen, NO reacts readily in a concentration-dependent manner to form peroxynitrite, a potent oxidant capable of oxidizing lipids and proteins. Peroxynitrite inactivates both α-1-antiprotease [16] and tissue inhibitors of metalloproteinases and may activate matrix metalloproteinases (MMPs) [17, 18]. Thus, NO exposure could predispose to lung matrix injury.

Azoulay et al. [10] reported emphysema-like changes in adult rats exposed to 2 ppm NO for 6 weeks. Holt and co-workers [11], using 10 ppm NO and longer exposures of up to 30 weeks, reported gross emphysematous changes in mice lungs, but only airspace enlargement when exposed to NO_2. More recently, Mercer et al. [12] reported emphysema-like destruction of the alveolar septa, with focal degeneration of lung interstitial cells, interstitial matrix and connective tissues, in adult rats exposed to 0.5 ppm NO, with twice daily 1-hour spikes to 1.5 ppm for 9 weeks. They also reported somewhat similar but less extensive changes using NO_2. These studies focused on chronic low-dose NO exposure as may occur with environmental pollution and involved only adult animals. Neonatal animals of several species exhibit delayed injury and more tolerance to hyperoxia than adults of the same species, therefore observations made in adult animals do not necessarily reflect the response in younger animals [19, 20]. To our knowledge, no report exists on the effects of short-term exposure to NO or hyperoxia, or both together as may occur during therapeutic uses, on the lung matrix of neonatal animals. Also, there are no reports on the effects of their combined exposure on the lung matrix in experimental animals of any age.

Because NO has pro-oxidant properties, we hypothesized that inhaled NO will result in biochemically detectable lung interstitial matrix and basement membrane injury during short-term exposure, and that these changes will be exacerbated by concurrent hyperoxia.

We measured lung lavage fluid proteins, hydroxyproline, hyaluronan and laminin as biochemical markers of lung matrix and basement membrane injury in piglets after 5 days' exposure separately in room air (RA), RA + NO, O_2 and O_2 + NO. Lung extravascular water and collagen content were also assessed. To evaluate NO- or O_2-induced effects on matrix-altering enzymes, lung MMP enzyme activity and MMP-2 messenger RNA expression were quantitated. Lastly, we measured lung collagen content in a subgroup of piglets exposed to RA + NO and O_2 + NO after allowing a 3-day recovery period in RA.

Methods

Animal Exposure and Sacrifice

Twenty-eight piglets, ages 8–15 days, were randomized to breathe either: (1) RA, (2) RA + 50 ppm, NO, (3) hyperoxia (O_2) ($FiO_2 \geq 0.96$) or (4) O_2 + NO for 5 days, then followed by sacrifice. In additional experiments, 10 piglets breathed RA + NO or O_2 + NO followed by a 3-day recovery period in RA prior to sacrifice for the study of lung collagen content. Exposure was performed in a 200-liter chamber at ambient temperature with the animals allowed free access to food and water. High-grade oxygen and NO (Puritan Bennett, Overland Park, Kans., USA) were routed by way of connectors and regulators to the inlet port of the exposure chamber. Humidified oxygen and NO were introduced at flow rates sufficient to maintain chamber concentrations at ≥96% and 45 ± 5 ppm, respectively, throughout the experiment. For 150-g soda lime canisters and 500 g activated charcoal canisters (Sigma, St. Louis, Mo., USA) were used to maintain both carbon dioxide (CO_2) and nitrogen dioxide (NO_2) concentrations at ambient air concentration and at 0–1 ppm. Chamber outlet port gas concentrations were monitored for oxygen (Hudson Medical, Temecula, Calif., USA), CO_2 (Model ABL30 Blood Gas Analyzer, Radiometer, Westlake, Ohio, USA), NO and NO_2 (Sensor Stik, NO Model 4584 and NO_2 Model 4586, Exidyne Instruments, Exton, Pa., USA).

At the end of the exposure period, anesthesia was accomplished with 2 mg/kg xylazine, 20 mg/kg ketamine and 40 mg/kg sodium pentobarbital prior to sacrifice. Euthanasia was accomplished with high-dose pentobarbital.

The lungs were removed promptly upon cessation of cardiac activity. Pieces of the left lower lobe were minced, then flash-frozen in liquid nitrogen and stored at –80°C until used for enzyme activity studies. The remaining left lower lobe was fixed in 4% formalin. The right lung was lavaged with 175 ml Dulbecco's saline, prewarmed to 37°C and the lavageate centrifuged at 300 *g* for 10 min to remove cellular contents [21]. The supernatant was then stored at –80°C until analyzed. Prior to the lavage, the right upper lobe segment was isolated and not lavaged. This segment was bronchially perfused with 4% formalin to a pressure of 24 cm H_2O. It was inflated at this pres-

sure for 24 h before being processed for alveolar septal width measurements.

This study was approved by the Institutional Animal Care Utilization Committee of the University of Missouri at Kansas City, Missouri.

Lavage Fluid Protein Assay

Lavage fluid total protein was assayed using a commercially available kit (BCA kit, Cat # B-9643, Sigma) according to the manufacturer's protocol. The molecular weight profile of lavage fluid protein was resolved by SDS-PAGE. Densitometric analysis of the albumin band (60 kD) was performed and the results expressed as the ratio of the band density in treated animals to that of the RA control animals.

Lung Water Assessment

Lung water content was assessed by previously reported methods [22]. Briefly, wet lung weight was obtained for both lungs, then a piece of the left upper lobe was dried in an oven for 5 days at 60°C after recording the wet weight. The percent lung water was then derived using the formula (wet lung weight – dry lung weight/wet lung weight × 100). Extravascular lung water content per lung DNA was derived after correcting the wet lung and dry lung weights for blood content.

Hydroxyproline Assay

Hydroxyproline in lung homogenate and lavage fluid was assayed according to the method of Reddy and Enwemeka [23]. Briefly, aliquots of standard hydroxyproline solution or test samples were mixed with an equal volume of 2 N sodium hydroxide to a total volume of 50 µl. The mixtures were then hydrolyzed by autoclaving at 120°C for 20 min. Chloramine-T 450 µl was then gently mixed with the hydrolysate, and the oxidation was allowed to proceed at room temperature for 25 min. 500 µl of freshly prepared Ehrlich's aldehyde reagent was then added to the hydrolysate, gently mixed, then incubated at 65°C for 20 min to develop the chromophore. The absorbance of each sample was read at 500 nm using a Gilford Response™ spectrophotometer and the hydroxyproline content calculated from the standard curve. All chemicals were from Sigma. Serum hydroxyproline was also measured by HPLC [24].

Alveolar Septal Width

Twenty random fields from the right upper lobe segment were examined (40×) on hematoxylin- and eosin-stained lung sections. The width of one septum in each field was measured with computerized image analysis (Media Cybernetics, Silver Spring, Md., USA) and the 20 measurements were averaged for each of the animals.

Hyaluronan Assay

Lung lavage fluid hyaluronan was assayed with a commercially available radiometric assay kit, Pharmacia HA (Pharmacia-Upjohn, Diagnostic Division), according to the manufacturer's directions. Briefly, to 100 µl of HA standard solution and test samples, 200 µl of HABP-^{125}I solution was added and the mixture incubated for 60 min at 4°C. 100 µl of HA-Sepharose was then added, followed by further incubation at 4°C for 45 min. After incubation, 2 ml of decanting solution was added, then the mixture was centrifuged for 10 min at 1,500 *g*. The tubes were decanted, then allowed to stand inverted on dry absorbent paper for 30 s. Bound radioactivity was measured in a gamma counter and hyaluronan content of samples was derived from comparison to the standard curve.

Laminin Assay

Laminin was quantified in the lung lavage fluid using a commercially available 'sandwich' ELISA kit (Chemicon, Temecula, Calif., USA) according to the manufacturer's protocol. Briefly, laminin strips in a plate holder were rehydrated with 200 µl wash buffer for at least 15 min at room temperature. Outside the plate, 50 µl of laminin standards or samples were incubated with an equal volume of rabbit anti-human laminin for 10 min at room temperature. Tests were performed in duplicate and the results assayed. 100 µl of each sample/standard and antibody mixture were then added to the plate after removal of wash buffer, followed by incubation for 60 min at room temperature. Plates were then washed 4 times with wash buffer. 100 µl of goat anti-rabbit IgG-HRP conjugate was added to each well, followed by incubation at room temperature for 30 min, then the wells were washed 4 times with wash buffer. The absorbance in each well was monitored at 540 nm in a microplate reader after addition of 100 µl TMB/E substrate solution until it reached 0.200, at which time the reaction was stopped by adding 25 µl 50% H_2SO_4 to each well. The plate was then read at 450 nm. The laminin concentrations in the samples were interpolated from the standard curve.

MMP Activity Assay

Frozen lung samples were weighed and homogenized in 20 vol 0.25% Triton X-100 in 0.01 *M* calcium chloride using PowerGen 125 (Fisher Scientific) for 30 s at maximum RPM. After centrifugation of the homogenate at 6,000 *g* for 20 min, the pellet was resuspended in 20 vol calcium chloride, and 0.15 *M* sodium chloride in 0.05 *M* Tris-HCl (pH 7.5). The suspension was heated at 60°C for 6 min, cooled and recentrifuged at 10,000 *g* for 20 min at 4°C. MMP activity was assayed in the supernatant and lung lavage fluid using a commercially available assay kit (Molecular Probes, Eugene, Oreg., USA) according to the manufacturer's protocol. The principle of the assay is based on MMP degradation of DQ Gelatin, a nonfluorescein conjugate, with resultant release of fluorescence. Thus, by incubating DQ Gelatin with samples of standard MMP, the sample MMP activity can be deduced from the standard curve. To 80 µl of reaction buffer in each well, 20 µl of 1 mg/ml DQ Gelatin solution was added, then gently mixed. 100 µl of purified *Clostridium* MMP or of the sample was then added to the reaction mixture and allowed to incubate for at least 2 h at room temperature, protected from light. Fluorescence intensity was measured in a microplate fluorescence reader. One unit of enzyme activity is defined as the amount of enzyme required to liberate 1 µ*M* L-leucine equivalents from collagen in 5 h at 37°C, pH 7.5.

MMP-2 mRNA Analysis

Total RNA was isolated using Ultraspec RNA reagent (Biotech Laboratories, Houston, Tex., USA) according to the manufacturer's instructions. A cDNA probe specific for MMP-2 mRNA was developed by amplifying a fragment of MMP-2 cDNA using polymerase chain reaction (PCR). Primers used for PCR were

5′-CTGGGCAACAAATATGAGAGCTGC-3′ and

3′-ACGGACCTTACGGTAGGGGCTATT-5′.

The reaction mixture was preheated at 95°C for 1 min. Denaturation, annealing and elongation temperatures were 96°C, 62°C and 72°C for 35 s, 2 min and 2 min, respectively. The reaction was run for 30 cycles followed by a final extension at 72°C for 10 min. The PCR product (782 bp) corresponding to nculeotides 1019–1800 in human MMP-2 cDNA [25] was purified using the QIAquick PCR purification kit (Quiagen, Santa Clarita, Calif., USA) and was radiolabeled using the DECAprime II DNA labeling kit (Ambion, Austin,

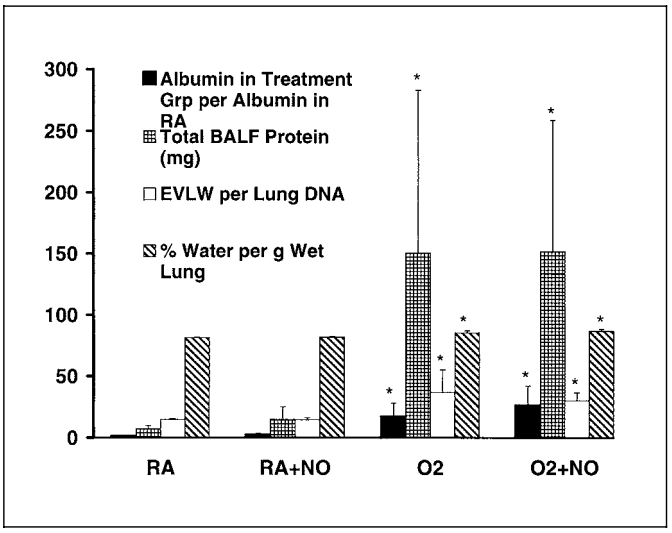

Fig. 1. Lung water and lavage fluid proteins in neonatal piglets after exposure to hyperoxia and inhaled NO. Lavage fluid albumin and total protein levels in O_2 and O_2 + NO groups were significantly elevated compared to RA controls, $p < 0.05$. Similarly, % water/gm wet lung and extravascular lung water (EVLW) content were significantly elevated in both groups compared to RA controls, * $p < 0.05$.

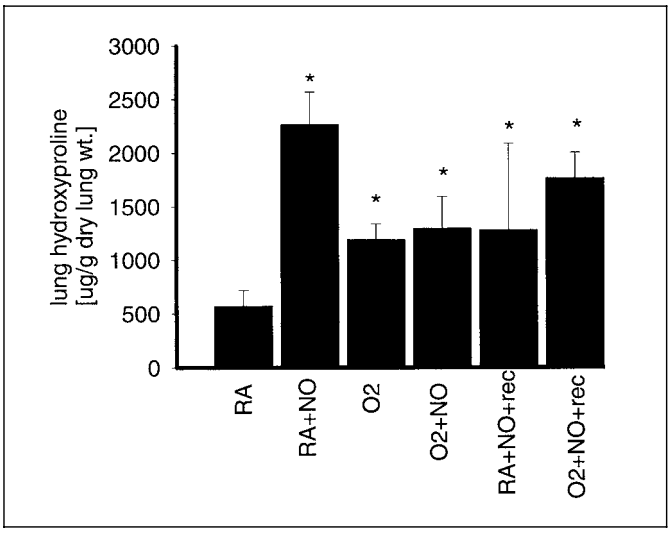

Fig. 2. Total lung hydroxyproline content in the experimental groups. After 5 days exposure, total lung hydroxyproline increased 400% in the RA + NO exposed piglets and 200% in the O_2 as well as O_2 + NO groups relative to the RA group, * $p < 0.05$. However, recovery in room air for 3 days decreased total lung hydroxyproline in the RA + NO piglets from 400% to only a 200% increase above RA piglets level. The O_2 and O_2 + NO groups did not decline.

Tex., USA). RNA samples (20 µg per lane) were electrophoresed on 1% agarose formaldehyde gel and transferred to positively charged nylon membranes (Boehringer Mannheim, Indianapolis, Ind., USA) to which the RNA was cross-linked by ultraviolet irradiation. Membranes were then prehybridized at 65 °C for 2 h. Hybridization to the ^{32}P-labeled probe was carried out overnight at 65 °C. Membrane washing was performed under standard conditions [26] prior to exposure to X-ray films. The autoradiograms were analyzed densitometrically by displaying the image and quantitating band intensities on a personal densitometer SI running the Image Quant software (Molecular Dynamics, Sunnyvale, Calif., USA). Results were normalized to 18S rRNA by rehybridizing the membranes to ^{32}P-labeled 18S rRNA probe (Ambion).

Statistics

Statistical analysis was performed using a computerized statistical software package, Instat (GraphPad Software, San Diego, Calif., USA). Data were analyzed with one-way analysis of variance (ANOVA). A p value <0.05 was considered statistically significant. Results were expressed as mean ± standard deviation (SD) unless otherwise noted.

Results

Albumin, Total Protein and Lung Water. Lung lavage fluid total protein and albumin were significantly elevated only in the O_2 and O_2 + NO piglet exposure groups compared to RA animals, $p < 0.05$ (fig. 1). There were no differences between RA + NO and RA exposed piglets. The extravascular lung water and percent lung water content was significantly elevated in the O_2 and O_2 + NO exposed piglets as compared to the RA group, $p < 0.05$ (fig. 1). The lung water content of RA + NO piglets was not different from that of the RA group.

Hydroxyproline Assays. Lung collagen expressed as hydroxyproline content per gram dry lung weight increased by 400% in the RA + NO group compared to the RA piglets ($p < 0.001$). After 3 days' recovery in RA the increase in lung collagen had declined from 400 to only 200% that of the RA piglets ($p < 0.05$). The O_2 and O_2 + NO groups showed a 200% increase relative to the RA control piglets ($p < 0.01$), but their hydroxyproline content was significantly less than that of the RA + NO group ($p < 0.05$). Lung collagen content in the O_2 + NO group remained elevated at 200% that of the RA piglets even after 3 days' recovery in RA (fig. 2). Mean lung lavage fluid hydroxyproline concentration was increased approximately 20-fold in the O_2 and O_2 + NO group relative to that in the RA group ($p < 0.05$) (fig. 3). However, no significant difference was evident between O_2 versus O_2 + NO groups. There was no difference between the RA + NO and the RA group. Hydroxyproline was not detectable in the serum of any of the experimental groups.

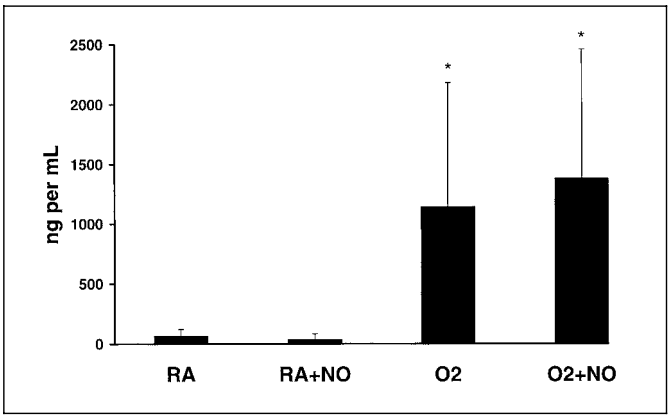

Fig. 3. Effect of hyperoxia and inhaled NO on lung lavage fluid hydroxyproline levels in neonatal piglets. Lavage fluid hydroxyproline levels in O_2 and O_2 + NO groups were significantly elevated compared to RA controls, * $p < 0.05$.

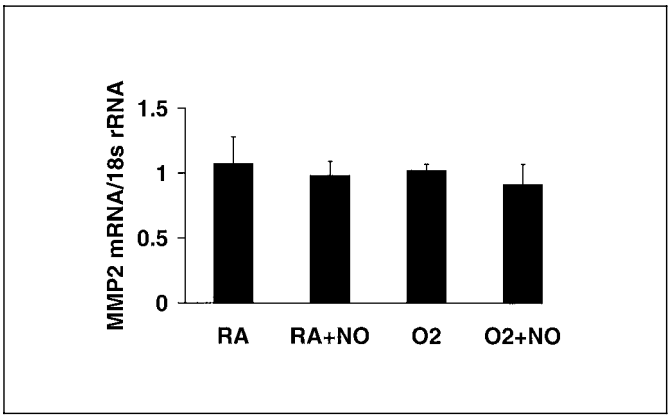

Fig. 4. Effect of hyperoxia and inhaled NO on lung MMP-2 mRNA expression. No significant differences were detected.

Alveolar Septal Width. Light microscopy showed inflammatory cell infiltrate, septal edema and tissue necrosis in the O_2, O_2 + NO and O_2 + NO + recovery piglet groups. The RA + NO and RA + NO + recovery groups were similar to the RA group. Mean alveolar septal width (mean ± SD μm) were significantly greater in the O_2 (12.99 ± 2.23), O_2 + NO (15.01 ± 2.92) and O_2 + NO + recovery (13.44 ± 1.60) compared to the RA (8.73 ± 1.55) group, $p < 0.01$. The RA + NO (10.80 ± 1.38) and RA + NO + recovery groups (11.70 ± 2.50) though tending to be higher, were not different from the RA group.

Hyaluronan. Hyaluronan was detected in the lung lavage fluid of all groups. Although hyaluronan tended to be higher in the O_2 and O_2 + NO groups, no significant difference was evident between the groups (table 1).

Laminin. Laminin also was detectable in lung lavage fluid from all groups. Mean lung lavage fluid laminin levels were not statistically different between the groups (table 1).

MMP Activity in Lung Homogenate and Lavage Fluid. Mean MMP activity per lung was significantly elevated in the O_2 and O_2 + NO exposed groups compared to the RA control group ($p < 0.05$) (table 2). However, no significant difference was detectable between the O_2 and the O_2 + NO group. Also, the RA + NO group values were not different from those of the RA group. MMP activity was not detectable in the lung lavage fluid.

MMP-2 mRNA. MMP-2 enzyme mRNA levels in the experimental groups are shown in figure 4. No significant differences were detected between any two of the four groups.

Table 1. Lavage fluid extracellular matrix contents

	RA	RA + NO	O_2	O_2 + NO
Laminin, ng/ml	84 ± 48	131 ± 89	127 ± 62	133 ± 59
Hyaluronan, μg/l	30 ± 16	31 ± 9	52 ± 28	61 ± 35

Values are mean ± SD; no significant difference detected.

Discussion

Pulmonary extracellular matrix degradation with concomitant development of emphysema-like lesions following prolonged exposure to oxidant gases has previously been reported [1–4, 27]. Hyperoxia, NO_2 and NO have all been implicated in this process in adult animals [1, 4, 11]. There are no reports in adult or neonatal animals on the effect of combined exposure to NO and hyperoxia on the lung matix. Also, no reports exist about the effects on the lung extracellular matrix of short-term inhaled NO use as is typical in therapeutic interventions.

In this report, we present the combined and individual effects of NO and hyperoxia on the neonatal pulmonary matrix using a piglet model. The results show that 5 days' exposure to RA + NO quadrupled, while O_2 and O_2 + NO only doubled total lung collagen in young piglets, compared to RA breathing. Allowing the animals to recover in RA for 3 days reduced the lung collagen increase in the RA + NO group by half, while that in the O_2 and O_2 + NO

Table 2. Gelatinase activity/physiologic data

	RA	RA + NO	O_2	O_2 + NO
Gelatinase activity, U/lung	565.60 ± 271.35	848.43 ± 309.24	982.17 ± 139.82*	1,075.90 ± 301.64*
Gelatinase activity, U/mg lung DNA	0.70 ± 1.06	4.73 ± 4.061*	5.70 ± 2.46*	4.026 ± 3.0*
Body weight at start of experiment, kg	3.38 ± 0.34	3.07 ± 0.19	3.34 ± 0.48	3.28 ± 0.67

Values are mean ± SD. * $p < 0.05$, significant difference from room air controls.

groups was unaffected. Alveolar septal width increased significantly only in the O_2 and O_2 + NO, but not in the RA + NO groups. Changes in the septal width are probably more reflective of the cellular swelling and edema occurring from exposure to hyperoxia. Unlike the piglets exposed to RA + NO, which did not show an increase in lung lavage fluid hydroxyproline levels in comparison to the RA group, the O_2- and O_2 + NO-exposed piglets had approximately a 20-fold increase in their levels. One explanation for these findings is that exposure to RA + NO increases collagen synthesis alone while O_2 and O_2 + NO increase both synthesis and degradation. This might explain the smaller increase in lung collagen content in the O_2 and O_2 + NO exposed piglets in comparison to those exposed to RA + NO. Indeed, Välimäki et al. [28], in their study of collagen metabolism in rat lungs during chronic intermittent hyperoxia, reported increased accumulation of lung collagen due to increases in the synthesis exceeding the rise in degradation. The result from the present study is compatible with their finding.

An elevated lung lavage fluid hydroxyproline level in adult animals exposed to hyperoxia has also previously been reported [1, 3]. Kerr et al. [2], using rat lung tissue slices, have demonstrated increased hydroxyproline release during exposure to hyperoxia which was attributed to oxidant-triggered proteolytic activity. Adamson et al. [3], in separate experiments using irradiation, bleomycin or hyperoxia, similarly reported increased lung lavage fluid hydroxyproline in adult rats. Riley et al. [1] reported increased lung lavage fluid hydroxyproline levels in adult rats after 60 h exposure to hyperoxia and emphysematous changes 9 weeks after exposure. In their study, Riley et al. [1] did not detect hydroxyproline in the serum, nor was desmosine, a marker for elastin degradation, detected in the lung lavage fluid. We did not detect hydroxyproline in the serum of any piglet group in the current study. Thus, it is unlikely that increased capillary permeability or elastin degradation would account for the presence of hydroxyproline in the lavage fluid. Furthermore, although hydroxyproline is present in elastin, it is an unlikely source for the increased hydroxyproline, as it is relatively more resistant to oxidant and proteolytic degradation compared to collagen [1, 2].

Hydroxyproline is present in small amounts in surfactant apoproteins, especially surfactant protein A. We did not detect any differences in lung surfactant protein A by immunohistochemical staining between the groups. Therefore, this would not account for the increased levels of hydroxyproline in the lung lavage fluid of the O_2 or O_2 + NO groups relative to the RA group. The pulmonary interstitial matrix contains mostly type I and III collagens whereas type IV collagens are found in the basement membrane [29]. We did not define which type of collagen accounts for the hydroxyproline in the lung lavage fluid but we speculate it is mostly types I and III collagen. This is because of their relative abundance and intimate contact with the alveolar epithelial and capillary endothelial cells, which are usually injured during hyperoxia [30]. The finding of increased hydroxyproline in the lung lavage fluid has important implications, since neonatal animals of several species have an apparent increased tolerance to hyperoxia, with delayed onset of pulmonary injury [19, 20]. Thus, the result suggests that biochemically detectable injury involving the lung matrix may be occurring even during this period of relative tolerance.

The apparent failure of NO to induce lung matrix injury in this model was somewhat unexpected, but may be related to NO's role as a 'molecular chameleon'. Thus NO, under differing conditions, may exhibit pro-oxidant or antioxidant properties, leading to unpredictability of its effects [7, 8]. The few reports of NO-induced pulmonary matrix damage, unlike reports of hyperoxic injury, utilized prolonged exposure periods, and relied upon morphometric and histological evidence without concurrent biochemical data. Thus, direct comparisons with the present study are not possible [10–12].

In addition to their elevated lung lavage fluid hydroxyproline level, the O_2 and O_2 + NO piglets, unlike those exposed to RA + NO, also had increased lavage fluid total protein and albumin levels, as well as increased lung extravascular water content. This would suggest that they suffered alveolar capillary basement membrane injury with resultant high vascular permeability.

We had expected to find increased laminin and hyaluronan levels in the lung lavage fluid as further evidence of basement membrane and pulmonary matrix degradation, respectively. Hyaluronan, laminin and type IV collagen are reportedly elevated in the bronchoalveolar lavage fluid of humans with pulmonary diseases associated with pulmonary matrix and basement membrane destruction [31, 32]. However, we detected only a statistically insignificant trend towards an increase of these biochemical markers in the lung lavage fluid of the experimental groups compared to the controls.

We found significantly increased MMP enzyme activity per lung in the lung homogenates from animals exposed to O_2 and O_2 + NO, relative to the RA exposure group. Interestingly enough, the same O_2 and O_2 + NO animal groups as discussed earlier had increased lung lavage fluid hydroxyproline concentration. A possible explanation would be that increased MMP activity contributes to the rise in the lavage fluid hydroxyproline, perhaps by degrading lung matrix collagens. Appropriate denominators for normalization of measured biochemical markers in whole lung experiments involving hyperoxia exposure are often difficult to determine since hyperoxia may significantly alter lung weight, protein and DNA content due to occurrence of pulmonary edema and cellular damage [33–37]. When results of the lung MMP enzyme activity are expressed in units of activity per mg lung DNA, the RA + NO, O_2 and O_2 + NO piglet groups show a significant difference relative to the RA group. Since these same piglet groups also had increased accumulation of lung collagen in comparison to the RA group, it would then appear that increased MMP activity may be related to increases in lung collagen accumulation. Increased MMP activity may be necessary for the tissue modelling occurring in the lungs in the presence of increased lung collagen accumulation [38].

The failure to detect significant differences in the levels of MMP-2 messenger RNA in the lungs of animal groups in this experiment, compared to the RA group animals, would suggest that upregulation of this particular MMP is not responsible for the increased enzyme activity reported. This finding may be consistent with our failure to detect significant differences in lavage fluid laminin concentration. Although other MMPs are known to have this capability, MMP-2 is a specific MMP for degrading type IV collagens, which together with laminins are found exclusively in basement membranes [38]. Thus, degradation of lung interstitial matrix types I and III collagens rather than basement membrane type IV collagens may be the predominant source of increased lavage fluid hydroxyproline. It is possible that the increased MMP activity noted may not necessarily involve de novo synthesis but rather may be due to increased activation of proenzyme forms or inactivation of tissue inhibitors of metalloproteinases and antiproteases. Devaskar et al. [39] have reported induction of type I and increased transcription of type IV (MMP-2) collagenases, as well as an increase in their activities following hyperoxic exposure. We did not specifically evaluate type IV collagenase activity, but the failure to detect an increase in MMP-2 messenger RNA may suggest that its activity remained unchanged in this experimental model. It is possible that this is due to differences in animal species, piglets versus rats, or age, neonatal versus adults.

The current study is the first to suggest that NO may have profibrotic properties, which raises the specter that NO use could potentially lead to development of pulmonary fibrosis. Although the dose of NO used in this study is high, a safe, effective dose of NO is yet to be defined: a recent report suggests that the use of very low doses of NO is not only ineffective in improving oxygenation in human infants with pulmonary hypertension, but prevents subsequent response to higher doses [40].

Specific features of this study include limiting NO exposure to a few days, inclusion of a recovery group in the study, elimination of NO_2, avoidance of confounding effects of underlying inflammation, or the use of positive pressure ventilation.

Limitations of this study include the use of only a high single dose of NO and O_2, single duration of exposure, and use of an animal model. The lack of endotracheal intubation with positive pressure ventilation differs from typical clinical settings of NO use but avoids confounding influences of mechanical ventilation. We used the single dose of 50 ppm NO in this study because we believed that any toxicity from NO was likely to occur at higher doses.

In summary, our findings suggest that short-term exposure to inhaled NO, O_2 or O_2 + NO increases lung collagen accumulation in neonatal piglets and that NO is the most effective. This may be because NO, unlike O_2 or O_2 + NO, does not induce a concurrent increase in pulmonary matrix degradation. Increased activity of MMPs other than MMP-2 appears to be involved in the observed effects.

The increase in lung collagen content found with NO exposure appeared potentially reversible as demonstrated by a significant decline after a 3-day recovery period in RA. The findings of increased lavage fluid proteins, hydroxyproline levels and increased lung MMP activity suggest that at least in the short term during concurrent use, high FiO_2 may be a more important determinant of pulmonary matrix injury than is exposure to NO. The increase in lung collagen accumulation observed with NO in this study represents a new finding which suggests that NO may have the potential to induce pulmonary fibrosis. Further experiments incorporating different doses and durations of exposure to NO and oxygen, as well as longer recovery periods as may obtain in the clinical setting are warranted to fully assess the implications of this finding.

Acknowledgments

Supported in part by NIH R-01 HL62079 and by a Physician Scientist Award from Children's Mercy Hospital (W.E.T.) and by the Katherine B. Richardson Foundation (I.I.E. and D.W.T.). The authors thank Kelly Carver for her secretarial assistance and patience in the preparation of the manuscript.

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