Inhaled nitric oxide improves lung structure and pulmonary hypertension in a model of bleomycin-induced bronchopulmonary dysplasia in neonatal rats

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Inhaled nitric oxide improves lung structure and pulmonary hypertension in a model of bleomycin-induced bronchopulmonary dysplasia in neonatal rats. Am J Physiol Lung Cell Mol Physiol 297: L1103–L1111, 2009. First published October 16, 2009; doi:10.1152/ajplung.00293.2009.—Whether inhaled nitric oxide (iNO) prevents the development of bronchopulmonary dysplasia (BPD) in premature infants is controversial. In adult rats, bleomycin (Bleo) induces lung fibrosis and pulmonary hypertension, but the effects of Bleo on the developing lung and iNO therapy on Bleo-induced neonatal lung injury are uncertain. Therefore, we sought to determine whether early and prolonged iNO therapy attenuates changes of pulmonary vascular and alveolar structure in a model of BPD induced by Bleo treatment of neonatal rats. Sprague-Dawley rat pups were treated with Bleo (1 mg/kg ip daily) or vehicle (controls) from day 2 to 10, followed by recovery from day 11 to 19. Treatment groups received early (days 2–10), late (days 11–19), or prolonged iNO therapy (10 ppm; days 2–19). We found that compared with controls, Bleo increased right ventricular hypertrophy (RVH), and pulmonary arterial wall thickness, and reduced vessel density alveolarization. In each iNO treatment group, iNO decreased RVH (P < 0.01) and wall thickness (P < 0.01) and restored vessel density after Bleo (P < 0.05). iNO therapy improved alveolarization for each treatment group after Bleo; however, the values remained abnormal compared with controls. Prolonged iNO treatment had greater effects on lung structure after bleomycin than late treatment alone. We conclude that Bleo induces lung structural changes that mimic BPD in neonatal rats, and that early and prolonged iNO therapy prevents right ventricle hypertrophy and pulmonary vascular remodeling and partially improves lung structure.

bronchopulmonary dysplasia (BPD) is the chronic lung disease of infancy that most commonly occurs in premature infants who require mechanical ventilation and oxygen therapy for acute respiratory distress, but it also occurs in immature infants with few signs of initial disease (25). BPD has a multifactorial etiology, including oxygen toxicity (40), preterm delivery (53), hypoxia or hyperoxia (32, 50), infection, and inflammation (25). Perinatal inflammation can result from diverse mechanisms, including chorioamnionitis, hyperoxia, infection, or ventilator-induced lung injury, and it is strongly related to the development of BPD in various animal models, as well as in the clinical setting (8, 29, 37, 59).

Histologically, BPD is characterized by disruption of alveolar and vascular growth after preterm birth. The saccular and alveolar periods of lung development are characterized by rapid increases in vessel growth and septation of the distal air space. These periods of lung development occur from the 24th wk of gestation through the first 3 yr of life in humans and during the first 3 wk of postnatal life in rodents (32). Inflammatory mechanisms contribute to the impairment of lung structure and pulmonary vascular development, resulting in pulmonary hypertension and right ventricular hypertrophy (32, 50).

Various animals models of BPD have been developed to improve our understanding of mechanisms that impair lung development and cause neonatal lung injury, including exposure to prolonged hyperoxia (4), intra-amniotic endotoxin exposure (55), mechanical ventilation (9, 15), neonatal monocrotaline (1), eNOS−/− mice (2, 5), and the use of angiogenesis and VEGF inhibitors (52). Each model provides insights into the pathobiology of BPD; however, the pathogenesis of BPD and interventions that may prevent the development of BPD remain poorly understood (25).

Past studies have suggested that impaired nitric oxide (NO) production may contribute to the pathogenesis of BPD (5, 30, 31, 48, 51, 63), and that inhaled NO therapy may improve lung vascular and airspace structure in diverse models of BPD (9, 26, 27, 30, 42, 51). However, the effects of iNO have been variable, with some studies reporting marked enhancement of lung structure and others reporting minimal effects (3, 9, 33, 42). Similarly, clinical trials suggest that iNO therapy may protect human preterm newborns from developing BPD (6, 24, 47), but others have not demonstrated significant differences in the risk for BPD after iNO therapy (21, 57). Mechanisms underlying the lack of consistency among these studies are unclear; whether these differences in outcomes reflect variability in dose, timing, or duration of therapy remain unclear and require further study (7).

Bleomycin (Bleo) administration has been used extensively to explore mechanisms of pulmonary fibrosis in adult rodents (12–14, 36). In adult rats, intratracheal bleomycin treatment induces an early inflammatory response during the initial 10 days after instillation, followed by progressive fibrosis and striking changes in lung structure over time (12, 14). Since BPD is characterized by early inflammation with late fibroproliferation, we studied the effects of bleomycin in neonatal rats to develop a potentially useful model for BPD. We report that systemic bleomycin treatment impaired vascular and alveolar growth and caused pulmonary hypertension in neonatal rats, which may provide an additional approach for studies of mechanisms relevant to BPD. Using this animal model, we also evaluated the effects of early and prolonged iNO therapy on lung structure after Bleo treatment. We specifically sought to
determine whether early, late, or prolonged iNO treatment would improve lung alveolar and vascular structure in neonatal lung injury induced by Bleo.

**METHODS**

**Animals.** All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased (Harlan Laboratories, Indianapolis, IN) and maintained at Denver’s altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen pressure [P{O}_2], 122 mmHg) for at least 1 wk before giving birth. Pups were delivered naturally (on day 21), and the litter size was maintained at 10–12 pups. Animals were fed ad libitum and exposed to 12:12-h light-dark cycles throughout the study period.

**Study design.** We designed this study to assess the effects of inhaled NO (iNO) treatment on pulmonary vascular structure and lung growth in a model of neonatal lung injury and pulmonary hypertension induced by daily Bleo injections. We evaluated whether iNO could prevent (early treatment concomitant with Bleo injections) or rescue (late treatment during the week of recovery after Bleo injections) lung structure from the adverse effects of Bleo. Furthermore, the combination of early and late iNO treatment was evaluated to assess the effects of prolonged therapy on Bleo-induced alterations of lung architecture.

Starting on day 2 of life, pups received Bleo (bleomycin sulfate) at a dose of 1 mg/kg in 0.9% saline (2 µg/g body wt by 30-gauge needle in the right iliac fossa) or 0.9% saline (vehicle control) by intraperitoneal injection for 10 days (see Fig. 1). Animals were placed in a Plexiglas chamber and exposed to room air with or without inhaled NO (10 ppm). O{2}, NO, and O{2} levels were monitored with an iNOVent controller (iNO Therapeutics, Clinton, NJ) with the exhaust directed into an outside air vent at 10 l/min. Four to twelve animals were studied within each of the five study groups. At 19 days of age, animals were killed for studies of pulmonary hypertension (by measuring right ventricular hypertrophy, RVH), pulmonary vascular structure and growth (by measuring pulmonary artery wall thickness and pulmonary artery density), and alveolarization (by measuring radial alveolar count, RAC). Western blot analysis was performed to compare lung protein expression for endothelial NO synthase (eNOS), vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2) between study groups.

**Fixation of lung tissue.** Animals were killed with intraperitoneal injections of pentobarbital sodium (100 mg/kg). Rat lungs were prepared and fixed in situ at the end of the study (day 19). PBS was infused into the main pulmonary artery through a right ventricular canula to flush the pulmonary circulation free of blood. A catheter was placed in the trachea, and the lungs were inflated at 25 cm H{2}O pressure with 4% paraformaldehyde in PBS and maintained under constant pressure for 1 h. This pressure was selected for fixation based on previous studies that demonstrated consistent and uniform lung inflation, and because the lung was fixed in situ, the chest wall contributes to lower compliance than found in lungs that are completely removed from the thorax. The trachea was ligated to maintain pressure, and the lungs were immersed in paraformaldehyde solution overnight. The left lower lobe was embedded in paraffin, and sections were cut with a microtome set at 5 µm and stained with hematoxylin and eosin. The investigator was blinded to the study group of the sections at the time of morphometric analysis.

**Right ventricular hypertrophy.** The heart was removed at autopsy, the right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed, and the ratio of RV to LV+S weights were determined as an assessment of RVH.

**Lung volume measurements.** Lung volume was determined by fluid displacement, as previously described (46). A beaker of water was...
placed on a balance and adjusted to zero. Excess of liquid was gently wiped from the lung and submerged while avoiding contact with the jar walls. The weight in grams corresponds to the volume of the organ in milliliters.

**Lung morphometric analysis.** Alveolarization was assessed by performing RAC by the method of Emery and Mithal (16). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was drawn to the edge of the acinus (as defined by a connective tissue septum or the pleura), and the number of septa intersected by this line was counted. Ten counts were performed for each animal. The intra-alveolar distance was measured as the mean linear intercept (MLI) by standard methods, as previously described (5, 54). Briefly, 6 images of each section were captured on a Zeiss Axioscope2 microscope, using the 10x objective and captured as a high-resolution PICT image by a QICAM digital camera (1,392 × 1,040 pixel resolution; Qimaging, Burnaby, Canada). Large airways or vessels were avoided. These images were processed with a plug-in that was previously developed by Dr. Balasubramaniam and Dr. Christopher Coulon, (5) to use ImageJ, a public-domain Java image-processing program created by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD (http://rsb.info.nih.gov/ij). MLI was determined by dividing the total length of 42 lines drawn across the lung section by the number of intercepts encountered.

Pulmonary artery wall thickness measurements were performed on small pulmonary arteries (30 – 80 μm) associated with terminal bronchioles and distal air spaces with a Zeiss Interactive Digital Analysis System (ZIDAS; Carl Zeiss, Thornwood, NY), as previously described (35, 51). At least 10 pulmonary arteries were measured for each animal. Wall thickness and external diameter were measured...
directly and expressed as the percentage of wall thickness, which was calculated with the following formula: \[
\frac{\text{external diameter} - \text{internal diameter}}{\text{external diameter}} \times 100
\]

Immunohistochemistry for factor VIII was performed to assess vascular density. Paraffin-embedded slides from formalin-fixed tissue were deparaffinized in xylene. The sections were rehydrated by serial immersions in 100% ethanol, 95% ethanol, 70% ethanol, and water. Sections were digested with proteinase K at a concentration of 50 \( \mu \text{g/ml} \) for 10 min at room temperature and then washed with PBS with 2.7 mM KCl, 1.2 mM KH\(_2\)PO\(_4\), 138 mM NaCl, and 8.1 mM Na\(_2\)HPO\(_4\). Endogenous peroxidase activity was reduced by immersion in 3% hydrogen peroxide in methanol. After rinsing, sections were covered in 10% horse serum for 1 h and incubated with rabbit anti-factor VIII antibody (1:200) diluted in PBS with 1% BSA overnight (#A0082 Dako USA Carpenteria, CA). After incubation, the sections were rinsed with PBS and incubated with horseradish peroxidase (HRP)-labeled secondary antibody diluted 1:2,000 in PBS with 1% BSA for 1 h. After incubation with the secondary antibody, the sections were rinsed with PBS, incubated in ABC complex (Vector Laboratories, Burlingame, CA) for 30 min at room temperature, rinsed in PBS, and developed with diaminobenzidine and hydrogen peroxide. Slides were lightly counterstained with hematoxylin. The slides were then dehydrated by sequential immersion in 70% ethanol, 95% ethanol, 100% ethanol, and xylene before applying coverslips. For each slide, 10 pictures were captured by digital camera for analysis. For assessment of vessel density, images of factor VIII-stained slides were captured with the 20× objective.

Western blot analysis. We performed Western blot analysis of distal lung homogenates from control, Bleo, and Bleo + early and late iNO study groups on day 19. Frozen lung samples were homogenized in ice-cold buffer containing 50 mM Tris·HCl (pH 7.4), 1 mM EDTA, 1 mM EGTA, and 1% Halt protease inhibitor single-use cocktail (Pierce Biotechnology, Rockford, IL). The samples were centrifuged at 1,500 \( g \) for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by the Bradford method (10), using BSA as the standard. Twenty-micrograms of protein sample/lane were resolved by SDS-PAGE, and proteins from the gel were transferred to PVDF membranes. Blots were blocked for 1 h in 5% nonfat dry milk in TBS with 0.1% Tween 20. These blots were incubated overnight at 4°C with either mouse anti-human polyclonal eNOS/NOSIII antibody (BD-610297, 1:500; BD Biosciences, Durham, NC), rabbit anti-human polyclonal VEGF antibody (SC-507, 1:500; Santa Cruz Biotechnology, Santa Cruz, CA), or rabbit anti-human polyclonal VEGFR-2 antibody (KDR/flk-1; SC-504, 1:250; Santa Cruz Biotechnology). Antibodies were diluted in 5% nonfat dry milk in TBS with 0.1% Tween 20. After being washed, blots were incubated for 1 h at room temperature with a goat anti-rabbit IgG-HRP antibody (SC-2054, 1:5,000; Santa Cruz Biotechnology), or goat anti-mouse IgG-HRP antibody. Bands were visualized by enhanced chemiluminescence (ECL Advance kit; Amersham Pharmacia Biotech, Buckinghamshire, UK). For Western blot analysis of VEGF, recombinant...
mouse VEGF was used as a control. Densitometry was performed using Quantity One (Bio-Rad Laboratories, Hercules, CA).

Statistical analysis. Statistical comparison was made by ANOVA and Fisher’s protected least significant difference test with the StatView software package (Abacus Concepts, Berkeley, CA). Data are presented as means ± SE. Differences were considered significant at \( P < 0.05 \).

RESULTS

Body weight, lung volumes. Sixty animals were studied for morphometric and Western blot analyses. Two animals died during the study: one in the Bleo-treated group and one in the Bleo + late iNO group. Compared with control rats raised in room air, body weights were significantly lower in all the study groups, but not different from each other \( (P < 0.05; \text{Fig. 2A}) \). Lung volume increased by 27% in the Bleo-treated group compared with the control group \( (P < 0.05; \text{Fig. 2B}) \). Early iNO and early plus late iNO-treated groups decreased lung volume by 28% compared with the Bleo-treated group \( (P < 0.05; \text{Fig. 2B}) \).

Right ventricular hypertrophy (RVH), vascular wall thickness, and vessel volume density. Bleo treatment increased RV/LV+S by 73% above values measured in control animals \( (P < 0.001; \text{Fig. 3}) \). For each study group, iNO treatment decreased RV/LV+S compared with Bleo-treated animals \( (P < 0.001; \text{Fig. 3}) \), achieving levels not different from controls \( (P < 0.001; \text{Fig. 3}) \). Vascular wall thickness was increased from 16.8 ± 1.9% in the control group to 37.2 ± 2.8% in the Bleo group \( (P < 0.001; \text{Fig. 4D}) \). In all groups, iNO treatment decreased wall thickness compared with Bleo-treated animals \( (P < 0.01; \text{Fig. 4}) \). However, only iNO treatment for 17 days (the early plus late iNO group) decreased wall thickness to similar values measured from control animals \( (20.4 ± 1.0%, \ P = 0.27 \) compared with control group; \text{Fig. 4D})

Vessel density was also measured in slides from each group (Fig. 5, A-C). Compared with controls, Bleo reduced vessel density by 47% \( \text{(control: 12.0 ± 1.5 vs. bleomycin: 6.4 ± 0.9 vessels/hpf, \ P < 0.001; Fig. 5D}) \). In each treatment group, iNO increased vessel density compared with Bleo-treated animals \( \text{(late iNO: 9.2 ± 1.7, early iNO: 8.1 ± 1.6, and early and late iNO: 9.7 ± 0.2 vessels/hpf; \ P < 0.05 vs. Bleo for each group; Fig. 5D}) \).

Lung histology and morphometrics. Compared with control animals, lung histology of animals exposed to Bleo was characterized by decreased septation, distal airspace enlargement, and a reduction in complexity (Fig. 4, A and B). These differences were assessed by morphometric measurements of RAC and MLI. RACs were markedly reduced by 47% in rats treated with Bleo compared with control animals \( \text{[from 8.8 ± 0.3 (control) to 4.6 ± 0.3 (Bleo), \ P < 0.001; Fig. 6A])} \). Early iNO and early plus late iNO-treated groups significantly increased RAC compared with the Bleo group \( (P < 0.05; \text{Fig. 6A}) \). However, iNO treatment did not increase RAC to levels close to control values in any of the study groups (late iNO: 5.3 ± 0.3; early iNO: 5.7 ± 0.3; and early plus late iNO: 6.3 ± 0.3; \ P < 0.05; \text{Fig. 6B}) \).

Parallel findings were observed for comparisons of MLI. Rats treated with Bleo had increased MLI compared with controls \( \text{[123 ± 3.9 \mu m (Bleo) vs. 91.2 ± 2.8 \mu m (controls), \ P < 0.001; Fig. 6B])} \). Early and early plus late iNO groups had significantly lower MLI compared with the Bleo group \( (P < 0.05; \text{Fig. 6A}) \), but MLI values remained higher than controls \( (P < 0.05; \text{Fig. 6B}) \).

Lung eNOS, VEGF, and VEGFR-2 protein expression. Lung eNOS protein content was not different between the control and Bleo groups. However, early plus late iNO treatment increased lung eNOS protein content by 89% and by 74% compared with the control and Bleo groups \( (P < 0.05 \) for each; \text{Fig. 7A}) \). Lung VEGF protein content was not different between the control and Bleo groups, but there was a statistically insignificant trend for increased VEGF protein in the prolonged iNO treatment group \( (P = 0.07; \text{Fig. 7B}) \). Lung VEGFR-2 protein content was also not different between groups (Fig. 7C).
In the current study, the treatment with INO prevented the development of pulmonary hypertension but did not completely restore lung architecture to normal after Bleo treatment. Differences between studies may be due to several factors, including the diversity of models and species, or differences in the timing and duration of INO therapy.

McNamara et al. (34) previously described the development of pulmonary hypertension in neonatal rats after Bleo treatment, but the impact of Bleo on lung structure, especially with regard to the distal airspace, were not assessed. We report that in addition to changes in the pulmonary vasculature, Bleo induced striking changes in the distal lung airspace, including reduced septation with increased distal airspace size and increased lung volume, along with decreased vascular density. These changes mirror structural lesions that occur in human infants with BPD, suggesting that Bleo treatment may provide a new model for studies of pathogenetic mechanisms underlying BPD. In addition, the previous study of Bleo treatment demonstrated that brief exposure to NO, as inhaled gas or with systemic administration of SIN as an NO donor, did not acutely lower pulmonary artery pressure (34). In contrast, fasudil, a rho kinase inhibitor, caused pulmonary vasodilation, suggesting a loss of vascular responsiveness to INO therapy in this model (34). Our findings show that early and prolonged INO treatment attenuates RVH, improves pulmonary artery wall thickness, and enhances vascular density. We speculate that brief INO treatment may be ineffective in causing acute pulmonary vasodilation in this model, but in addition, INO may be more effective in this model during early and more prolonged treatment, before extensive pulmonary vascular structural changes occur. Mechanisms that may alter acute reactivity to INO with established pulmonary hypertension in this model are not understood, but may include high Rho-kinase (ROCK) activity (34); downregulation or impaired activity of the NO target, soluble guanylate cyclase (sGC), increased type 5, cyclic GMP-specific phosphodiesterase (PDE5); or other changes in pulmonary arterial smooth muscle cells (18, 23, 58). Bleo is known to induce oxidative stress in experimental studies (12–14), which may decrease INO responsiveness due to the presence of oxidized sGC or upregulation of PDE5 (18, 23, 58). Alternatively, the lack or responsiveness to INO in the previous study may be due to poor INO delivery within the lung due to...
We observe a trend of increased VEGFR-2 expression in the MMP9/tissue inhibitor of metalloproteinase 1 balance (62). Overexpression of eNOS in the endothelium has also been shown to attenuate Bleo-induced lung fibrosis by altering the MMPs ratio (63). Previous studies have shown that pulmonary hypertension develops in adult rats after Bleo treatment, as reflected by increased pulmonary artery pressure, right ventricular hypertrophy, and increased vessel wall thickness (22, 45, 60). Future studies measuring changes in sGC and PDE5 activities and expression after Bleo and that compare the effects of iNO with sGC activators and PDE5 antagonists may provide further insight into differences underlying these studies.

Previous studies of adult rodents have shown that Bleo, generally administered directly into the airway, causes striking lung injury with progressive fibrosis (12–14, 36). An intratracheal Bleo injection, macrophages, neutrophil and lymphocytes counts in bronchoalveolar fluid, as well as lung inflammatory and mitogenic mediators (ET-1, PDGF-β, MCP-1, and TNF-α) will increase within 1 wk. After the first week, lung hydroxyprolin content and expression of profibrogenic markers will increase (12, 13, 41). In contrast, systemic Bleo treatment of neonatal rat pups caused minimal signs of fibrosis but markedly reduced lung alveolarization and vascular growth. These differences were further illustrated by the increase in lung volume after Bleo, which suggests higher compliance than controls, and not the low lung compliance observed after pulmonary fibrosis in adult rats (20, 41). Mechanisms through which Bleo impairs lung architecture and induces pulmonary hypertension in neonatal rats are not known, but differences in these responses may reflect the mode of delivery (ip vs. airway) or developmentally unique responses to this injury.

Several studies have examined mechanisms of Bleo-induced lung injury in adult rats that are related to oxidant-mediated DNA damage and release of fibrogenic cytokines (14, 17, 22, 62). Recent studies have shown that Bleo alters NO-cGMP signaling in the adult lung. Hennes et al. (22) found that inhibition of PDE5, which increases cGMP, improves lung structure. Intratracheal bleomycin administration in mice induced high levels of ROCK and RhoA activities. After PDE5 treatment, the ROCK and GTPase RhoA activities were partially inhibited by an increase of PKG related to the high level of cGMP (22). We found that iNO therapy increased eNOS protein content in bleomycin-treated rat pups, but mechanisms underlying this effect are unknown. Bland et al. (9) previously observed that eNOS protein content of intrapulmonary arteries in chronically ventilated preterm lamb was higher with iNO treatment, compared with preterm lamb chronically ventilated without iNO. Potential mechanisms through which NO may improve lung structure in this model, include a decrease in Bleo-induced apoptosis (17) and a reduction of the expression and activation of matrix metalloproteinases (MMPs), extracellular matrix-degrading zinc-dependent enzymes (62). Alternatively, iNO may reduce oxidative stress, inducing DNA-damaging (61), apoptosis (51) and production of cytokines such as TNF-α that are known to downregulate eNOS expression (39). Indeed, overexpression of eNOS in the endothelium has also been shown to attenuate Bleo-induced lung fibrosis by altering the MMP9/tissue inhibitor of metalloproteinase 1 balance (62). We observe a trend of increased VEGFR-2 expression in iNO-treated rats, a finding previously noted in the restoration of lung structure by iNO in eNOS-deficient mice recovering from neonatal hypoxia (5).

Past studies have also shown that pulmonary hypertension develops in adult rats after Bleo treatment, as reflected by increased pulmonary artery pressure, right ventricular hypertrophy, and increased vessel wall thickness (22, 45, 60). We found that Bleo increased RVH and impaired vascular structure after 10 days of daily Bleo intraperitoneal injections followed by 1 wk of recovery. McNamara et al. (34) in a neonatal model using the same dosage for 14 days also found an increased RVH and pulmonary vascular remodeling. These changes in vascular structure were associated to an increase in the activation of the GTPase RhoA and ROCK activity (34).

We conclude that Bleo treatment of neonatal rats induces lung structural changes, including reduced alveolar and vascular growth, and pulmonary hypertension, which are best improved by treatment with early and prolonged iNO therapy. We speculate that Bleo treatment of neonatal rats provides a useful model for studying mechanisms related to BPD and may be particularly helpful to explore questions underlying the striking variability of results from the multicenter randomized trials of iNO for the prevention of BPD in human preterm neonates.

DISCLOSURES

No conflicts of interest are declared by the authors.

REFERENCES


