Nitric Oxide Inhalation Decreases Pulmonary Artery Remodeling in the Injured Lungs of Rat Pups


Abstract—Vascular injury causes the muscularization of peripheral pulmonary arteries, which is more pronounced in the infant than in the adult lung. Although inhaled NO gas attenuates pulmonary artery remodeling in hypoxic rats, whether or not it protects the lung by mitigating vasoconstriction is unknown. This investigation tested whether inhaled NO decreases the muscularization of injured pulmonary arteries in rat pups by modulating vascular tone. One week after monocrotaline administration, the percentage of muscularized rat pup lung arteries was increased by ∼3-fold. Nevertheless, monocrotaline exposure did not cause right ventricular hypertrophy, pulmonary hypertension, or vasoconstriction. In addition, it did not increase the expression of markers of inflammation (interleukin-1β, intercellular adhesion molecule-1, and E-selectin) or of platelet-mediated thrombosis (GPIIb). Continuous inhalation of 20 ppm NO gas prevented the neomuscularization of the pulmonary arteries in pups with lung injury. Moreover, a 3-fold increase in cell proliferation and 30% decrease in cell numbers in pulmonary arteries caused by monocrotaline exposure was prevented by NO inhalation. These data indicate that inhaled NO protects infants against pulmonary remodeling induced by lung injury by mechanisms that are independent of pulmonary tone, inflammation, or thrombosis. (Circ Res. 2000;87:140-145.)

Key Words: inhaled nitric oxide ▪ pulmonary hypertension ▪ proliferation ▪ congenital heart disease ▪ bronchopulmonary dysplasia

Pulmonary vascular disease is an important complication of lung injury in congenital heart disease (CHD). In infants with ventricular septal defects, d-transposition of the great arteries, and atroventricular canal defects, increased left-to-right shunting of blood across the cardiac lesion causes pulmonary vascular injury. Vascular disease is also observed in the lungs of premature infants with chronic lung disease. Through mechanisms that are incompletely understood, lung injury is associated with the proliferation of smooth muscle cells (SMCs), or their precursors, in the walls of normally nonmuscular peripheral pulmonary arteries (a process known as neomuscularization). Because of restricted blood flow and increased tone in the muscularized arteries, the pulmonary vascular resistance (PVR) and arterial pressure increase, and right ventricular (RV) hypertrophy and failure are observed.

The structural changes observed in pulmonary vascular disease are recapitulated in animal models of lung injury. Treatment of rats with monocrotaline, a cytotoxic compound derived from the seeds of Crotalaria spectabilis, causes endothelial cell injury and neomuscularization of pulmonary arteries. The vascular injury is associated with serum leakage into the subendothelial space, inflammation, increased cytokine and growth factor expression, and cell proliferation. In adult rats, the remodeling of lung arteries leads to pulmonary hypertension and RV hypertrophy.

NO signaling is disrupted in injured pulmonary arteries. Endothelial cells synthesize NO using NO synthase, oxygen, and L-arginine. NO maintains endothelial cell integrity and decreases adhesion molecule expression. After entering the blood vessel lumen, NO decreases platelet aggregation and thrombus formation. NO that diffuses into SMCs decreases vascular tone and cell proliferation. It is likely that diminished endogenous NO signaling in children with lung injury promotes abnormal pulmonary vascular reactivity and remodeling. By inhalation, NO is delivered to the lung, where it increases cGMP levels and selectively dilates constricted pulmonary arteries. Although inhaled NO attenuates pulmonary artery remodeling in hypoxic infant and adult rats, its protective mechanisms are unknown. Because both pulmonary vasoconstriction and remodeling are observed in hypoxic rats and inhaled NO is a potent pulmonary vasodilator, it could attenuate neomuscularization by decreasing a remodeling stimulus associated with pulmonary vasoconstriction. In addition, NO could inhibit cell prolifer-
tion by modulating pulmonary inflammation and thrombosis. The purpose of our studies is to test whether or not inhaled NO mitigates vascular disease in the injured developing lung. In addition, whether the protective effect of inhaled NO requires pulmonary vasodilatation, inflammation, and thrombosis is examined.

**Materials and Methods**

**Pulmonary Hemodynamics and RV Weight**

Arterial pressures were determined using 6 to 7 Sprague-Dawley rat pups 9 days old in each experimental group, 1 and 2 weeks after subcutaneous monocrotaline (60 mg/kg) or PBS treatment. In anesthetized, mechanically ventilated pups, pressures were measured using cannulas secured in the carotid artery and in the main pulmonary artery.

In situ pulmonary artery flow and pressure relationships were determined using the lungs of 6 to 7 pups in each experimental group at 1 and 1.5 weeks after monocrotaline or PBS treatment. Pup lungs were ventilated mechanically and perfused using a peristaltic pump and HBSS containing indomethacin, albumin, and dextran. An incision in the left atrium permitted perfusate drainage. The RV weight was measured using 8 pups in each experimental group at 1 and 2 weeks after monocrotaline or PBS treatment.

**Analysis of Pulmonary Artery Structure**

The pulmonary artery muscularization was quantified in 4 pups in each experimental group at 7 days after treatment with PBS or monocrotaline and with and without 20 ppm continuously inhaled NO gas, as previously described. After pulmonary vein ligation, the arteries and airways were perfusion-fixed with formaldehyde. Miller staining of elastin in plastic-embedded sections permitted identification of arteries. The percentage of muscularized arteries, which contained 2 elastic laminae in their wall, was determined in the periphery of 3 lung lobes from each pup.

Using bromodeoxyuridine (BrdU) labeling and immunohistochemistry, the pulmonary artery cell proliferation was measured in 4 pups in each experimental group at 5 days after treatment with PBS or monocrotaline with and without inhaled NO. After BrdU administration, the pulmonary arteries were perfused with an emulsion of barium sulfate, gelatin, and phenol, and the lung was fixed with formaldehyde. Newly synthesized DNA was detected using an anti-BrdU antibody. To determine the lineage of the proliferating PA cells, additional sections were double-labeled with anti-α smooth muscle actin and anti-BrdU antibodies.

**Detection of Cytokine and Adhesion Molecule Expression**

Pulmonary interleukin (IL)-1β, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) gene expression were measured using RNA blot hybridization and RNA extracted from the lungs of 2 or 3 pups per experimental group 7 days after monocrotaline or PBS exposure. In situ pulmonary artery pressure-flow relationships and PVR were observed to be similar in the monocrotaline- and PBS-treated groups 1 week after PBS treatment. *P<0.05 vs groups 1 week after PBS or monocrotaline treatment and 2 weeks after PBS treatment.

**Statistics**

Data are mean±SD and were compared using a factorial model of ANOVA, and a Scheffé F test was used post hoc. Significance was determined at *P<0.05.*

An expanded Materials and Methods section is available online at http://www.circresaha.org.

**Results**

One week after treatment with monocrotaline, the pups were active and indistinguishable from those treated with PBS. However, 2 weeks after monocrotaline exposure, the pups had a lower body weight and PaO₂ in comparison with PBS-treated pups at 1 and 2 weeks and monocrotaline-treated pups after 1 week (*P<0.05).**

**Monocrotaline Exposure Causes Pulmonary Artery Remodeling in the Absence of Hypertension**

One week after treatment, when pulmonary artery neomuscularization is observed, monocrotaline did not cause pulmonary artery hypertension in pups (Figure 1). Furthermore, monocrotaline did not increase pulmonary artery tone, because acute inhalation of 20 ppm NO did not decrease the lung pressure in these pups (data not shown). Because cardiac output and, therefore, PVR could not be accurately measured in the pups, pulmonary artery pressure-flow relationships were determined using in situ perfused pup lungs. The pressure-flow relationships and PVR were observed to be similar in the monocrotaline- and PBS-treated groups 1 week after treatment (Figure 2). In addition, 1 week after treatment, the RV weight did not differ in pups treated with monocrotaline or PBS (Figure 3). Two weeks after treatment, however, monocrotaline exposure increased pulmonary artery pressure in the pups by nearly 70% (Figure 1). Additionally, the pressures measured in perfused lungs from monocrotaline-treated pups after 1.5 weeks were greater than those in lungs from pups treated with PBS at 1 or 1.5 weeks and in the lungs of monocrotaline-treated pups at 1 week (Figure 2). The PVR was also increased by 1.5 weeks in the monocrotaline-treated pups in comparison with control pups (PVR at 50 mL·kg⁻¹·min⁻¹: PBS-treated pups, 0.16±0.00, and monocrotaline-treated
Monocrotaline Does Not Induce Pulmonary Inflammation or Thrombosis in Rat Pups

Although monocrotaline exposure induces pulmonary inflammation\(^5,25\) and thrombus formation\(^26\) in adult rats, it is unknown whether they are observed in the remodeling pup lung. The lungs of pups 3.5 and 7 days after monocrotaline exposure did not exhibit increased adventitial and alveolar cellularity or cytokine and adhesion molecule expression (Figure 4). Inspection of monocrotaline-exposed pup lung sections did not reveal a decrease in the density of barium-gelatin–filled pulmonary arteries that would be observed with sections did not reveal a decrease in the density of barium–gelatin–filled pulmonary arteries that would be observed with exposure to monocrotaline. Data are mean ± SD; SDs of the PBS-treated pup lungs at 1.5 weeks are too small to be evident. \(^*\)P<0.05 vs other treatment groups.

Inhaled NO Prevents Monocrotaline-Induced Pulmonary Artery Remodeling

Monocrotaline increased the proportion of muscularized arteries in the alveolar duct and wall of pup lung by >3-fold 1 week after monocrotaline treatment (Figure 5). Importantly, continuous inhalation of NO gas protected the lungs from monocrotaline-induced pulmonary artery remodeling. Because monocrotaline exposure increases pulmonary artery cell proliferation in adult rats,\(^8,9\) we tested whether attenuated neomuscularization in NO-treated lungs was associated with decreased pulmonary artery cell proliferation. The increased cell proliferation in the walls of monocrotaline-treated pulmonary arteries was associated with NO inhalation (Figure 6). Because monocrotaline exposure is associated with SMC proliferation,\(^8,9\) double-immunolabeling of BrdU and smooth muscle actin was performed to determine whether the proliferating cells had an SMC lineage. Although monocrotaline exposure induced proliferation of epithelial cells in airways and endothelial and adventitial cells in peripheral pulmonary arteries, no multiplying SMCs were observed in the lung periphery after 5 days (Figure 7). Additionally, despite the increased cell multiplication, fewer cells per pulmonary artery were observed in the monocrotaline-treated pups than in the PBS-treated ones (8.2 ± 2.0 versus 6.4 ± 1.3; P<0.05). Importantly, inhaled NO was observed to prevent monocrotaline-induced pulmonary artery cell loss. Because monocrotaline exposure increases cell proliferation and yet decreases the cell numbers, these data suggest that it increases cell turnover in peripheral pulmonary arteries.

Discussion

Inhaled NO mitigates pulmonary artery remodeling in hypoxic newborn\(^19\) and adult rats\(^20,21\) through mechanisms that are incompletely understood. Although NO could attenuate pulmonary neomuscularization by directly inhibiting cell proliferation in hypoxic rats, it could indirectly influence remodeling by modulating pulmonary hypertension.
pups, we examined whether inhaled NO decreases lung artery neomuscularization in the absence of increased pulmonary tone. Monocrotaline exposure of pup lungs was observed to induce pulmonary artery neomuscularization and cell proliferation in the absence of pulmonary hypertension, inflammation, and thrombosis. Nevertheless, continuous inhalation of low levels of NO reduced the remodeling of the injured pup lungs. These data indicate that inhaled NO protects the developing lung from neomuscularization associated with vascular injury most likely via direct modulation of pulmonary artery cell proliferation.

Increased NO and cGMP signaling decreases rat lung injury.19–21,27–29 Nevertheless, data suggest that inhaled NO does not prevent pulmonary vascular remodeling in adult rats weeks after monocrotaline exposure.30,31 The reason why inhaled NO protects the lungs of pups but not of adult rats is unknown. However, studies indicate that the pathologic response to monocrotaline differs markedly in infant and adult rats.24 In addition, it is also possible that inhaled NO protects in the pup lung because the NO-cGMP signaling system is modulated during pulmonary development. The gene expression of soluble guanylate cyclase, an important receptor for NO, is highest in the newborn and infant lung and decreases to very low levels in the adult rat.32 Of note, treatment with L-arginine28 and phosphodiesterase inhibitors27 decreases pulmonary artery remodeling in adult rats after monocrotaline exposure. It is unknown why these agents that modulate systemic and pulmonary NO-cGMP signaling are effective in adult rats, whereas inhaled NO is not. However, the nonselective nature of these compounds suggests that they protect the lung by modulating systemic factors that contribute to pulmonary artery injury, such as inflammation and thrombosis.

Although NO inhibits cell replication in vitro34 and in systemic vessels in vivo,29 few studies have examined whether it has antiproliferative activity in the lung. In the present investigation, NO inhalation inhibited monocrotaline-induced cell proliferation in peripheral pulmonary arteries. In the pup lung, as in the adult,8,9 monocrotaline exposure induced proliferation of endothelial cells. However, in contrast with observations in adult rats,8,9 monocrotaline treatment caused proliferation of adventitial cells in pup pulmonary arteries. Because inhaled NO inhibits cell proliferation and the neomuscularization of pup lung arteries, it likely decreases the proliferation and differentiation of adventitial SMC precursors or the migration of these cells into the pulmonary artery wall. Because the differentiation of pulmonary adventitial cells into SMCs contributes to the neomuscularization of lung arteries in children,2 the inhibition of this process by inhaled NO is likely to be important in the attenuation of pulmonary vascular disease.

Inhaled NO probably attenuates lung remodeling by acting on cells residing in or transiting through the lung because inactivation of intravascular NO by hemoglobin33 inhibits its systemic effects. Inhaled NO may reduce neomuscularization by directly decreasing pulmonary cell proliferation, because NO-cGMP signaling inhibits the mitogen-activated kinase34–37 and cell cycle–regulatory systems in vitro.38 Additionally, inhaled NO may indirectly decrease neomuscularization by modulating vascular wall stress or growth factors.
released by cells transiting the lung. For example, because NO relaxes constricted vessels, it is possible that inhaled NO protects the lung through causing vasodilation. In support of this hypothesis are the observations that other vasodilators can prevent pulmonary vascular remodeling. However, inhaled NO protected the monocrotaline-treated pup lung from remodeling in the absence of vasoconstriction. Moreover, given that NO decreases leukocyte adhesion and platelet aggregation, it is possible that its protective mechanism requires the prevention of leukocyte and platelet-induced injury. However, we observed that inhaled NO protects the monocrotaline-treated pup lung in the absence of pulmonary artery inflammation and thrombosis. Although it is possible that NO decreases vascular remodeling in other models of injury through modulating vascular tone, inflammation, and thrombosis, our studies indicate that the salutary effect of inhaled NO in the injured newborn lung does not require these mechanisms.

The observation that inhaled NO decreases neomuscularization in the injured pup lung has important implications for the treatment of pulmonary vascular disease. Although corrective surgery in the neonatal period prevents abnormal pulmonary artery remodeling in many patients with CHD, it is associated with greater risks than if it is performed in older patients. In premature infants, no therapies have been identified to prevent the vascular complications associated with chronic lung disease or bronchopulmonary dysplasia. Therefore, it is desirable to identify therapies that will safely attenuate neomuscularization in the lung. Although the therapeutic potential of vasodilators has been explored, they only modulate vascular tone after neomuscularization has occurred. In addition, vasodilator therapy is not selective for the lung and may cause systemic hypotension, right-to-left shunting of blood across the cardiac lesion, and severe systemic hypoxemia. Because inhaled NO decreases pulmonary artery neomuscularization without requiring increased vascular tone in pups, it may have importance in preventing pulmonary artery remodeling disease in infants and children with lung injury. Furthermore, inhaled NO does not cause systemic vasodilatation, it decreases right-to-left shunting, and chronic inhalation is safe in the developing lung. The data presented herein suggest that clinical studies of the protective effect of inhaled NO should be performed in infants and children at risk for pulmonary vascular remodeling disease.

In summary, our studies demonstrate that inhaled NO protects injured lungs of rat pups from pulmonary artery remodeling before the onset of pulmonary hypertension. Because pulmonary vascular remodeling precedes pulmonary hypertension in many forms of CHD, inhaled NO therapy may play an important role in preventing vascular disease.

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References


Figure 7. Monocrotaline exposure and proliferation of pulmonary cells. Lungs of monocrotaline-exposed pups were perfusion-fixed with 4% phosphate-buffered formaldehyde, embedded in paraffin, and sectioned. After blocking endogenous peroxidases, lungs were incubated with an anti-α smooth muscle actin antibody (αSMA). After indirect immunohistochemical detection of α smooth muscle antibody with Vector Red substrate, and exposure to HCl to denature the DNA and strip the anti-αSMA antibody, sections were treated with anti-BrdU conjugated with peroxidase and 3,3′-diaminobenzidine. Lung sections were examined using bright-field microscopy after hematoxylin staining. Lung injury induced by monocrotaline treatment caused proliferation of pulmonary artery adventitial cells (small arrow) and endothelial (*) and epithelial (large arrow) cells. No pulmonary artery cells expressing αSMA, which is indicative of an SMC lineage, were observed to proliferate in response to monocrotaline exposure in pup lungs.


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