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 lungs 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 (GPIba). 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 atrioventricular 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-
ation 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 cannulae 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 a buffer 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 with and without 20 ppm continuously inhaled NO. In anesthetized, mechanically ventilated pups, pressures were measured using cannulae secured in the carotid artery and in the main pulmonary artery.

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Detection of Cytokine and Adhesion Molecule Expression
Pulmonary interleukin (IL)-1β, E-selectin, and intercellular adhesion molecule-1 (ICAM-1) gene expression was 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. Inspection of ethidium-stained gels confirmed equal loading of RNA samples. GPlha expression was detected in sections of pup lungs exposed to monocrotaline or PBS using a specific antibody (a gift from Dr Stefan Janssens, University Hospital Gasthuisberg, Leuven, Belgium) and immunohistochemistry.

Statistics
Data are mean±SD and were compared using a factorial model of ANOVA, and a Scheffe 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·min⁻¹·kg⁻¹: PBS-treated pups, 0.16±0.00, and monocrotaline-treated

Figure 1. Effect of treatment with monocrotaline (solid bars) or PBS (open bars) after 1 and 2 weeks on mean pulmonary arterial pressure (A), mean systemic arterial pressure (B), ratio of pulmonary to systemic arterial pressures (C), and heart rate (D). In the infant rat, monocrotaline-induced lung injury did not cause pulmonary hypertension until after 1 week of exposure. Data are mean±SD; n=6 to 7 per group. †P<0.05 vs groups 1 week after either PBS or monocrotaline treatment and 2 weeks after PBS treatment; ‡P<0.05 vs the group 2 weeks after PBS treatment.
pups, 0.21±0.02 mm Hg · kg⁻¹ · min⁻¹ · mL⁻¹; P<0.05 versus 1 week and versus each other). Two weeks after treatment with monocrotaline, the RV weight was nearly 45% greater than that observed in pups 1 and 2 weeks after PBS treatment (Figure 3). Together, these data indicate that pulmonary artery remodeling in pups 1 week after exposure to monocrotaline occurs in the absence of pulmonary hypertension.

**Monocrotaline Does Not Induce Pulmonary Inflammation or Thrombosis in Rat Pups**

Although monocrotaline exposure induces pulmonary inflammation²⁵ and thrombus formation²⁶ 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 thrombosis. In addition, immunoreactivity for GPIIa, a glycoprotein observed in platelet-rich thrombi,²³ was not detected in monocrotaline-treated lung pulmonary arteries (data not shown). These data indicate that pulmonary artery remodeling in the pup up to a week after monocrotaline exposure is not associated with lung inflammation or thrombosis.

**Discussion**

Inhaled NO mitigates pulmonary artery remodeling in hypoxic newborns¹⁹ and adult rats²⁰,²¹ 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. In rat
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 nonspecific 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 vitro14 and in systemic vessels in vivo,26 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,7 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\textsuperscript{27,39,40} 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\textsuperscript{41–43} and platelet aggregation,\textsuperscript{44,45} 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.\textsuperscript{46} 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 vasodilation; it decreases right-to-left shunting, and chronic inhalation is safe in the developing lung.\textsuperscript{47–49} 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


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