Maturational changes in the regulation of pulmonary vascular tone by nitric oxide in neonatal rats

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Submitted 22 June 2006; accepted in final form 5 September 2007

Chicoine LG, Paffett ML, Girton MR, Metropoulus MJ, Joshi MS, Bauer JA, Nelin LD, Resta TC, Walker BR. Maturational changes in the regulation of pulmonary vascular tone by nitric oxide in neonatal rats. Am J Physiol Lung Cell Mol Physiol 293: L1261–L1270, 2007. First published September 7, 2007; doi:10.1152/ajplung.00235.2006.—Nitric oxide (NO) is an important regulator of vasomotor tone in the pulmonary circulation. We tested the hypothesis that the role NO plays in regulating vascular tone changes during early postnatal development. Isolated, perfused lungs from 7- and 14-day-old Sprague-Dawley rats were studied. Baseline total pulmonary vascular resistance (PVR) was not different between age groups. The addition of KCl to the perfusate caused a concentration-dependent increase in PVR that did not differ between age groups. However, the nitric oxide synthase (NOS) inhibitor Nω-nitro-L-arginine augmented the K⁺-induced increase in PVR in both groups, and the effect was greater in lungs from 14-day-old rats vs. 7-day-old rats. Lung levels of total endothelial, inducible, and neuronal NOS proteins were different between groups; however, the production rate of exhaled NO was greater in lungs from 14-day-old rats vs. 7-day-old rats. Lung levels of both soluble guanylyl cyclase and cGMP were greater at 14 days than at 7 days. Vasodilation to 0.1 μM of the NO donor spermine NONOate was greater in 14-day-lungs than in 7-day lungs, and lung levels of both soluble guanylyl cyclase and cGMP were greater at 14 days than at 7 days. Vasodilation to 100 μM of the cGMP analog 8-(4-chlorophenylthio)guanosine-3′,5′-cyclic monophosphate was greater in 7-day lungs than in 14-day lungs. Our results demonstrate that the pulmonary vascular bed depends more on NO production to modulate vascular tone at 14 days than at 7 days of age. The observed differences in NO sensitivity may be due to maturation increases in soluble guanylyl cyclase protein levels.

Nitric oxide synthase; pulmonary vascular resistance; isolated perfused lungs; soluble guanylyl cyclase; guanosine-3′,5′-cyclic monophosphate

NITRIC OXIDE (NO) facilitates the transition from the high-resistance low-flow fetal pulmonary circulation to the low-resistance high-flow pulmonary circulation found shortly after birth (1, 15, 35). Studies examining the ontogeny of endothelial nitric oxide synthase (eNOS) in the developing rat lung found that eNOS protein levels and mRNA levels are low in early gestation, increase severalfold in late gestation, and are greatest at the time of birth (29, 38, 49). Following birth, there is a decrease of eNOS protein and mRNA to the low levels found in the adult rat. A similar pattern of eNOS expression has also been described in the developing porcine lung by Hislop et al. (25) and in the developing ovine lung by Halbower et al. (23).

Thus it is likely that elevated neonatal eNOS facilitates the transition from the high-resistance fetal pulmonary circulation to the low-resistance postnatal pulmonary circulation. Both, in vivo and in vitro studies have shown that eNOS expression is upregulated by several factors, including shear stress (39) and oxygen exposure (19, 31, 34). Furthermore, we have found that the effect of chronic hypoxia on eNOS expression is different in the neonatal vs. the adult rat lung (13, 40). In the neonatal rat lung, eNOS protein levels decreased with chronic hypoxic exposure, whereas in the adult rat lungs eNOS protein levels increased with this stimulus (13). These data suggest that eNOS protein expression is differentially regulated in the neonate vs. the adult. However, the effect of development during the neonatal period on eNOS activity and NO production is less well understood. Patients in the neonatal intensive care unit are at risk for developing pulmonary hypertension after birth, usually as a consequence of chronic lung disease or chronic pulmonary overcirculation (2, 4, 8, 9, 32, 33). Thus understanding how maturation of pulmonary vasomotor tone is regulated by eNOS ontogeny and NO production in the neonatal period is of prime importance in developing therapeutic strategies for preventing neonatal pulmonary hypertension. We hypothesized that NO regulation of pulmonary vascular tone changes during early postnatal development.

The regulation of vascular tone by NO may involve any component of the NO-cGMP pathway. NO is produced by the oxidation of L-arginine by NO synthases (NOS), of which there are three isoforms: neuronal (nNOS or NOS1), inducible (iNOS or NOS2), and eNOS (or NOS3). The NO produced by NOS can then diffuse to activate vascular smooth muscle soluble guanylyl cyclase (sGC). Activated sGC produces cGMP from GTP, and cGMP formed from sGC in the vascular smooth muscle cell results in protein kinase G-mediated vasodilation (6, 26). cGMP is metabolized to GMP by phosphodiesterases (PDE), of which there are multiple isoforms, with PDE5 found abundantly in the lung. Thus, to explore our hypothesis, we performed the following studies in isolated, perfused lungs from 6- to 8-day-old (hereafter referred to as 7 day old) and 13- to 15-day-old (hereafter referred to as 14 day old) Sprague-Dawley rats. We examined pulmonary vascular responses to non-receptor-mediated K⁺-induced vasoconstriction in the presence and absence of the nonspecific NOS inhibitor Nω-nitro-L-arginine (L-NNA). In addition, we compared whole lung total NOS isoform protein levels and exhaled nitric oxide in neonatal rats.
NO (exNO) production rates. To examine the sensitivity of the pulmonary vasculature to NO, we exposed preconstricted isolated lungs to the NO donor spermine NONOate and measured vasodilatory responses. We determined baseline levels in whole lung homogenates of cGMP, eNOS, and PDE5 at 7 and 14 days of life. Finally, to examine downstream effectors of NO-mediated vasodilation, we exposed preconstricted isolated 7- and 14-day-old lungs to 100 μM of the cGMP analog 8-pCPT-cGMP.

**METHODS**

Neonatal Sprague-Dawley rats (Harlan, Indianapolis, IN) were housed with their birthing dams. The animals were provided with fresh food, water, and clean bedding three times per week. All animals were housed on a 12:12-h light-dark cycle. All protocols employed in this study were reviewed and approved by both Institutional Animal Care and Use Committees of the University of New Mexico Health Sciences Center and of the Columbus Children’s Research Institute.

**Isolated, Perfused Lung System**

Lungs were isolated and perfused as previously described (14) but modified to accommodate the small size of 7- and 14-day-old rat pups. Briefly, rat pups at either 7 or 14 days of age were killed with a lethal dose of pentobarbital sodium (100 mg/kg ip). The animal was secured to a heating block to maintain body temperature at 38°C. The trachea was cannulated via a tracheotomy with custom-fitted polyethylene tubing (PE-90; Becton-Dickinson, Sparks, MD) that was connected to a mouse ventilator (Hugo/Saks), and the lungs were ventilated using a tidal volume of 5 ml/kg body wt and a rate of 50 breaths/min, using a gas mixture consisting of 5% CO₂ in room air. A median sternotomy was performed, and 5 units of heparin (total volume 0.05 ml) were injected directly in the right ventricle. A blood sample was obtained for determination of hematocrit (Hct). The pulmonary artery was cannulated with custom-fitted polyethylene tubing (PE-90; Becton-Dickinson). The preparation was immediately perfused at a rate of 0.1 ml/min with a physiological saline solution (PSS) containing (in mM): 129.8 NaCl, 5.4 KCl, 0.83 MgSO₄, 19 NaHCO₃, 1.8 CaCl₂, and 5.5 glucose with 4% albumin (wt/vol) and 300 μM meclofenamate (all from Sigma, St. Louis, MO) via a Masterflex roller pump (model 7524–10). Meclofenamate was dissolved in normal saline and then calibrated to a double-occlusion pressure (Pd) that approximates capillary perfusion resistance at 3 mmHg and end-expiratory pressure at 1 mmHg. Pulmonary arterial (Pₐ), venous (Pᵥ), and airway pressures were recorded continuously using a Dell PC, Codas data acquisition software and hardware (Dataq), and a Gould four-channel chart recorder. Inflow to the lung and outflow from the lung were simultaneously occluded as previously described (14). The arterial and venous pressures rapidly equilibrated to a double-occlusion pressure (Pₒ) that approximates capillary pressure in the lung (14, 17, 42). Total pulmonary vascular resistance (PVR) was calculated as (Pₐ - Pᵥ)/Q, pulmonary arterial resistance as (Pₐ - Pₒ)/Q, and pulmonary venous resistance as (Pᵥ - Pₒ)/Q.

**exNO Measurement In Isolated Perfused Lungs**

exNO was measured as previously described (14). Briefly, rat pups at either 7 or 14 days of age were killed with a lethal dose of pentobarbital sodium (100 mg/kg ip), intubated, and mechanically ventilated using room air and a mouse ventilator (Hugo/Saks) at a constant tidal volume of 5 ml/kg body wt and a respiratory rate of 50 breaths/min. Cannulas were placed in the pulmonary artery and left ventricle, and the lungs were perfused with PSS for a 30-min equilibration period. Exhaled gas was collected for 5 min in a mylar balloon attached to the ventilator exhaust port. The gas collected in the mylar balloon was analyzed using a chemiluminescence NO analyzer (Sievers, Boulder, CO). The exNO production rate in picomoles per minute was calculated using the minute ventilation (Vₑ × RR, where Vₑ is tidal volume and RR is respiratory rate) and the exNO concentration (ppb), as previously described (11, 17). The analyzer was calibrated using a standard curve generated daily with authentic NO (1 ppm in N₂; Matheson, Chicago, IL) mixed with NO-free N₂ using precision flowmeters to obtain concentrations ranging from 0 to 500 ppb (14, 18). The NO detection limit was 0.5 ppb (vol/vol).

**Immunoblot Analysis**

Immunoblot analysis was carried out as previously described (13, 14, 40). Lungs from 7- to 14-day-old rats were harvested, snap-frozen in liquid nitrogen, and stored at −80°C. Frozen lungs were ground in a precooled mortar and pestle and then homogenized on ice in Tris buffer (10 mM Tris-HCl (pH 7.4) containing 255 mM sucrose, 2 mM EDTA, 12 μM leupeptin, 1 μM pepstatin A, 0.3 μM aprotinin, and 1 mM phenylmethylsulfonyl fluoride (all from Sigma)). A commercially available protein assay (Bio-Rad, Hercules, CA) was performed on spun-clarified (1,500 g at 4°C for 10 min) supernatants to ensure equal gel loading. Samples (40 μg) were resolved by SDS-PAGE with 7.5% acrylamide along with molecular-weight standards (Bio-Rad) and an appropriate NOS standard (BD Transduction Laboratories, San Jose, CA). The separated proteins were transferred to polyvinylidene difluoride membranes (Bio-Rad) and blocked overnight at 4°C with 5% nonfat milk, 3% BSA (Sigma), and 0.05% Tween 20 (Bio-Rad) in a Tris-buffered saline solution (TBS) containing 10 mM Tris-HCl and 50 mM NaCl (pH 7.5). Rinsed blots were incubated for 4 h at room temperature with a mouse monoclonal antibody raised against iNOS, nNOS, eNOS, nNOS, SCG, or PDE5 (1:2,500; BD Transduction Laboratories) in TBS. Immunolabeling was achieved by incubation for 1 h at room temperature with horseradish peroxidase-conjugated goat anti-mouse IgG (1:10,000; Bio-Rad) in TBS. Chemiluminescence labeling was performed per kit instructions (GE Healthcare Biosciences, Piscataway, NJ). Protein bands were detected by exposure to chemiluminescence-sensitive film and quantitated by densitometric analysis (Sigma Gel; Jandel Scientific, San Rafael, CA). To control for protein loading, the blots were then stripped using a stripping buffer (each 100 ml contained 6.25 ml 1 M Tris·HCl, pH 6.8, 20 ml 10% SDS, 0.7 ml β-mercaptoethanol, and 73 ml double-distilled H₂O). The blots were reprobed for β-actin (1:10,000; Abcam, Cambridge, MA).

**Pulmonary and Circulating Levels of cGMP**

cGMP levels under basal conditions were determined by enzyme immunoassay (EIA) in 12 neonatal rats (n = 6 in each group of 7-day-old and 14-day-old pups; Cayman Chemical, Ann Arbor, MI) per the manufacturer’s instructions. Plasma proteins were ethanol precipitated for 5 min at room temperature and separated by centrifugation at 1,500 g for 10 min at 4°C. The supernatant was transferred to a clean microcentrifuge tube and dried under a stream of N₂ gas.
The role of NO production in K⁺-induced vasoconstriction. The K⁺-induced vasoconstriction experiment described above was repeated in isolated perfused lungs from 7 (n = 8) and 14 (n = 6)-day-old rat pups, with the exception that 300 µM of the NOS inhibitor L-NNA was present in the perfusate.

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<th>Age /± L-NNA</th>
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Fig. 1. Total pulmonary vascular resistance (PVR) and segmental vascular resistances were not different between age groups whether in the presence or absence of nitric oxide synthase (NOS) inhibition with 300 µM Nω-nitro-L-arginine (L-NNA). However, NOS inhibition resulted in a ~3-fold greater basal PVR in isolated lungs from both 7- and 14-day-old rat pups. A: baseline total PVR in isolated physiological saline solution (PSS)-perfused lungs from 7-day-old and 14-day-old rat pups measured in the presence (7 day, n = 5; 14 day, n = 5) or absence (7 day, n = 8; 14 day, n = 6) of L-NNA. B and C: segmental vascular resistance in isolated PSS-perfused lungs from 7-day-old and 14-day-old rat pups in the presence and absence of L-NNA in arteries (B) and veins (C). Data are means ± SE. The PSS included 5.4 mM KCl and 300 µM meclofenamate.
NO sensitivity. The sensitivity of the pulmonary vasculature in isolated perfused lungs to NO was assessed by adding (0.1 and 1.0 μM) the NO donor spermine NONOate to the perfusate in lungs isolated from 7-day-old (n = 6) and 14-day-old (n = 7) rat pups that were preconstricted with 30 mM KCl.

cGMP responsiveness. The sensitivity of the pulmonary vasculature to cGMP was assessed by adding (1 μM or 100 μM) the cGMP analog 8-(4-chlorophenylthio)guanosine-3',5'-cyclic monophosphate (8-pCPT-cGMP) to the perfusate in lungs isolated from 7-day-old (1 μM, n = 6; 100 μM, n = 6) and 14-day-old (1 μM, n = 3; 100 μM, n = 3) rat pups that were preconstricted with 30 mM KCl. 8-pCPT-cGMP was dissolved in normal saline at a concentration of 10 mM and stored at -20°C until thawed for use.

RESULTS

The role of NO production in baseline PVR in lungs from 7- and 14-day-old pups

At 7 days of age, rat pups weighed approximately one-half that of rat pups at 14 days (18.6 ± 0.8 grams at 7 days vs. 36.4 ± 1.3 grams at 14 days). Hct of 7-day-old pups were similar to those of 14-day-old pups (30.3 ± 0.8% at 7 days vs. 29.4 ± 0.8% at 14 days). Baseline PVR did not differ between age groups under basal conditions (Fig. 1A). The inclusion of 300 μM L-NNA in the perfusate resulted in an approximately threefold increase in basal PVR in isolated lungs from both 7-day and 14-day-old rat pups (Fig. 1A). The L-NNA-induced increase in PVR was due to a nearly threefold increase in arterial resistance (Fig. 1B) and a nearly threefold increase in venous resistance (Fig. 1C).

Fig. 2. The addition of KCl to the perfusate resulted in concentration-dependent increases in PVR. A: K+ resulted in a concentration-dependent increase in total PVR, and there was no difference in the K+-induced increase in total PVR between age groups. B: the K+-induced increase in segmental PVR was due entirely to arterial constriction, and K+ had no affect on venous resistance (C) in isolated lungs from neonatal rats. D: inhibition of NO production with 300 μM L-NNA augmented the total vasoconstrictor response to K+, and the K+-induced vasoconstriction was greater in lungs from 14-day-old pups than in lungs from 7-day-old pups. E: the K+-induced increase in PVR was all due to arterial constriction, and the K+-induced increase in arterial resistance was substantially greater in lungs from 14-day-old pups than in lungs from 7-day-old pups. F: K+ had no affect on venous resistance in isolated lungs from neonatal rats when L-NNA was added to the perfusate. Data from 7-day lungs (without L-NNA, n = 5; with L-NNA, n = 8); ○, data from 14-day lungs (without L-NNA, n = 5; with L-NNA, n = 6). Data are means ± SE. *14 day different from 7 day at same K+ concentration, P < 0.05. KCl was added to achieve final perfusate concentrations of 15, 30, and 45 mM.
However, even after L-NNA treatment, the major contributor to the total PVR continued to be arterial resistance (Fig. 1).

**The Role of NO Production in K⁺-Induced Vasoconstriction in Lungs from 7- and 14-Day-Old Pups**

Lungs isolated from both 7- and 14-day-old rat pups demonstrated similar concentration-dependent increases in PVR due to the addition of K⁺ (Fig. 2A). The vasoconstrictor response to K⁺ occurred only in the arterial bed in both lungs isolated from 7-day-old and 14-day-old pups (Fig. 2B). There was no change in venous resistance at any of the tested concentrations of K⁺ in lungs isolated from either 7-day-old or 14-day-old pups (Fig. 2C).

Addition of K⁺ resulted in pulmonary vasoconstriction in L-NNA-treated isolated lungs from both 7-day-old and 14-day-old rat pups (Fig. 2D). Lungs from 7- and 14-day-old rat pups demonstrated approximately three- and sixfold greater PVR response to K⁺, respectively, when L-NNA was included in the perfusate compared with the K⁺ response without L-NNA (Fig. 2, A vs. D). Furthermore, total and arterial responses to 30 and 45 mM K⁺ were greater in lungs from 14-day-old vs. 7-day-old pups following NOS blockade (Fig. 2, D and E). There was no venous response to K⁺ in lungs isolated from either 7- or 14-day-old rat pups (Fig. 2F). These results suggest that pulmonary NO production is involved in attenuating the K⁺ constrictor response at both ages and that pulmonary NO production plays a greater role in modulating this response at 14 days of age than at 7 days of age.

**Developmental Analysis of NO Production and NOS Expression**

To determine if age-related differences in NO production were involved in the different responses to K⁺ after L-NNA treatment, NO production rates in isolated perfused lungs were determined. We found that the production rate of exhaled NO from 14-day-old pups was significantly greater in the lungs isolated from 14-day-old pups than in the lungs from 7-day-old pups (Fig. 3).

**NO Sensitivity**

To determine if age-related differences in sensitivity to NO were involved in the different responses to K⁺ after L-NNA treatment, the response of preconstricted isolated lungs to spermine NONOate was determined. Lungs were preconstricted with 30 mM KCl, and 300 μM L-NNA was included in the perfusate. We found that baseline constricted PVR was not different between the age groups (28.8 ± 4.4 mmHg·min⁻¹·ml⁻¹ at 7 days vs. 33.6 ± 4.5 mmHg·min⁻¹·ml⁻¹ at 14 days). We also found that isolated lungs from both 7- and 14-day-old rat pups vasodilated in a concentration-dependent manner with the addition of spermine NONOate to the perfusate (Fig. 5). However, at 0.1 μM, there was significantly greater vasodilation in the lungs from 14-day-old rat pups than in the 7-day-old group (Fig. 5). This suggests that there is greater vascular NO sensitivity at 14 days of age than at 7 days of age.
Pulmonary Expression of sGC and PDE5 in the Lung

NO sensitivity is due at least in part to the level of cGMP generated in the vascular smooth muscle in response to NO. Cellular levels of cGMP are determined both by the levels of sGC and PDE5 in the lung. Therefore, the protein levels of sGC and PDE5 were determined by immunoblotting of lungs from 7- and 14-day-old rat pups. Interestingly, there were greater sGC protein levels in lungs from 14-day-old rat pups compared with the lungs from 7-day-old rat pups (Fig. 6A). However, there was no statistically significant difference in PDE5 protein levels between lungs from the two age groups (Fig. 6B).

**cGMP Responsiveness**

To determine if downstream modulators of NO are involved in the age-related differences in sensitivity to NO observed in this study, we tested pulmonary vascular sensitivity to cGMP in isolated perfused lungs. The cGMP analog 8-pCPT-cGMP (1 and 100 μM) was added to the perfusate in lungs isolated from 7- and 14-day-old rat pups that were preconstricted with 30 mM KCl. 8-pCPT-cGMP was used in these studies because it is a potent activator of cGMP-dependent protein kinases, has increased membrane permeability, and is metabolically stable toward PDEs (11, 28). The PVR of the preconstricted vasculature before the addition of 8-pCPT-cGMP was not different between the 7- and 14-day-old isolated lungs (8.52 ± 1.13 and 10.37 ± 0.82 mmHg·ml⁻¹·min⁻¹, respectively). The addition of 1 μM 8-pCPT-cGMP resulted in a small and similar vasodilatory response in both 7- and 14-day isolated lungs (Fig. 7). The addition of 100 μM 8-pCPT-cGMP to the perfusate resulted in a greater vasodilatory response in both 7- and 14-day isolated lungs compared with the 1 μM dose, but the response in the 7-day-old lung was greater than that observed in the 14-day lung (Fig. 7).

**Pulmonary and Circulating Levels of cGMP**

NO activates sGC, which can result in an increase in cGMP levels. Therefore, to determine if the presence of increased sGC protein levels without alterations in PDE5 protein levels were associated with greater cGMP levels, lung tissue and plasma from nonconstricted rat pups were assayed for cGMP content. We found that both pulmonary (Fig. 8A) and plasma (Fig. 8B) levels of cGMP were greater in the 14-day-old group compared with the 7-day-old pups.
Immunochemistry

To assess whether the findings from immunoblots on whole lung tissue homogenates were relevant to the pulmonary vasculature, immunohistochemistry was performed on lung tissues harvested from 7- and 14-day-old animals. In Fig. 9 representative images show that both enzymes are present in the pulmonary vascular wall at both ages studied [Fig. 9, A and B (PDE5) and D and E (sGC)].

DISCUSSION

The main findings of this study were that 14-day-old rat pups displayed 1) an enhanced vasoconstrictor response to K+ after NOS inhibition, 2) greater exNO production in the isolated perfused lung, 3) a greater response to low concentrations of spermine NONOate, 4) elevated lung sGC protein, 5) greater lung and plasma levels of cGMP than did 7-day-old rat pups, and 6) a smaller vasodilatory response to the membrane-permeable cGMP analog 8-pCPT-cGMP. Our results demonstrate a greater vasodilatory influence of NO at 14 days than at 7 days of age. This appears to be due in part to greater pulmonary NO production at 14 days. Furthermore, the pulmonary circulation becomes more sensitive to NO from day 7 to day 14, which maybe due to enhanced cGMP production secondary to elevated sGC expression. Taken together, these results are consistent with our hypothesis that NO regulation of pulmonary vascular tone changes during early postnatal development.

The majority of the PVR in these buffer-perfused neonatal rat lungs was located in the arterial circulation. Thus the majority of the pressure drop across the lung occurs upstream of the pulmonary capillary bed, with the result that capillary pressure is maintained at a relatively low level. We have previously found in buffer-perfused isolated lungs from adult rats that the PVR was fairly evenly distributed between arteries and veins (41, 43). Thus there may be developmental changes in the pulmonary arterial bed that continue from 14 days until adulthood and result in a lowering of the contribution of the arterial bed to the total PVR. This concept is consistent with studies demonstrating that the pulmonary arterial bed remodels after birth, and the remodeling is characterized by a decrease in wall thickness and a resultant increase in lumen diameter (3).

Inhibition of NO production resulted in greater basal PVR in the neonatal rat lung. This is consistent with our previous studies in isolated perfused neonatal pig lungs (18, 37). Furthermore, in isolated perfused lamb lungs, NOS inhibition increased basal perfusion pressure (21). Thus basal NO production is involved in the maintenance of the normally low PVR in the neonatal lung. In contrast to neonatal lungs, we have previously found that NOS inhibition had no effect on basal PVR in isolated perfused adult rat lungs (40). Furthermore, we have previously found that expression of eNOS protein was substantially less in adult rat lungs compared with neonatal lungs (13). Taken together, these results suggest that...
NO is involved in the maintenance of basal pulmonary vascular tone in the neonate, but not in the adult.

The vasoconstrictor response to $\mathrm{K}^+$ was not different between the two age groups and occurred essentially entirely in the arterial bed. An arterial site of action for $\mathrm{K}^+$-induced constriction is consistent with previous studies in isolated lungs from adult rats (20, 48). However, when the production of NO was inhibited, $\mathrm{K}^+$ caused a greater increase in arterial resistance in 14-day lungs than in the 7-day lungs. This finding suggests that the pulmonary circulation of the 14-day-old rat is more dependent on NO production in modulating vasoconstrictor responses than that of the 7-day-old rat. Pulmonary vascular tone is a balance between constrictor tone and dilator tone. The mechanisms responsible for the increased dependence on NO for vasodilator tone may include alterations in the production of other vasodilators. Cyclooxygenase products, including PGI, PGD, and PGE, have been implicated as vasodilators in neonatal lungs (22, 46). However, meclofenamate was included in the perfusate in the current experiments, thereby eliminating possible influence of these products in our study. In addition, there may be alterations in the production of vasoconstrictors during neonatal development. For example, endothelin-1 (ET-1) is a potent vasoconstrictor that has been found to regulate pulmonary vasomotor tone. Levy et al. (30) found that expression of ET-1 decreased, whereas expression of NOS increased after birth in neonatal piglets. However, Jankov et al. (27) found that, although lung prepro-ET-1 mRNA levels decreased significantly between birth and 4 days in rats, there was no difference in lung prepro-ET-1 mRNA levels between 7 and 14 days (27). Thus it is unclear what role other modulators of pulmonary vascular tone play in the difference in pulmonary responses to $\mathrm{K}^+$ after NOS inhibition in isolated lungs from neonatal rats.

NO production increased from 7 days to 14 days in the isolated rat lung in the absence of a change in protein levels of any of the NOS isoforms in whole lung homogenates. One

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Fig. 9. Immunohistochemistry images of lung sections from 7-day-old and 14-day-old rats. Representative lung sections are shown that were probed for sGC at 7 days (A) and 14 days (B) that demonstrate positive staining in the vascular wall (arrows). Lung sections probed for PDE5 at 7 days (D) and 14 days (E) demonstrate positive staining in the vascular wall (arrows). Nonimmune IgG was used to probe similarly prepared lung sections as controls for nonspecific antibody staining (C and F).
explanation is that total lung NOS activity may be enhanced during this period. This is consistent with a study by Arrigoni et al. (5) wherein calcium-dependent NOS activity increased from 6 days to 14 days of age in neonatal pig lungs. The regulation of eNOS activity is complex and includes availability of substrate and cofactors, as well as chaperone and scaffolding proteins, and phosphorylation of various residues (16). Thus further studies are needed to determine the mechanisms underlying the apparent increased total lung NOS activity found in 14-day-old rats. We chose to measure exNO production as a measure of total lung NO production. Although measurement of exNO does not differentiate airway vs. vascular sources of NO, we have previously found that in neonatal pigs the measurement of exNO production is a sensitive, rapidly responding measure of physiologically relevant total lung NO production (12). However, our use of whole lung homogenates limits our interpretation of these findings because of limitations in detecting changes in NO production and/or NOS levels localized to specific anatomic locations, such as lung epithelial cells and/or pulmonary vascular endothelial cells.

Vasodilatory sensitivity to NO was also greater at 14 days than at 7 days of age. This enhanced sensitivity was associated with elevated tissue and plasma cGMP levels in this group.

One potential mechanism for an increase in cellular cGMP levels is a greater expression of sGC in the 14-day-old animals. Indeed, we observed greater sGC protein expression by immunoblot analysis in this group compared with lungs from 7-day-old rats. In addition to finding more sGC in whole lungs isolated from 14-day-old pups, we also found abundant sGC protein expression in the pulmonary vascular wall by immunohistochemistry. Recently, Moreno et al. (36) found that expression of lung sGC mRNA and protein increased from 3 to 18 h of age from 14 days of age in piglets and that this increase in sGC protein expression levels was associated with greater relaxation to an NO donor. Similarly, Behrends et al. (7) reported that sGC protein levels and activity were greatest in the neonatal rat when compared with fetal and adult rats. In contrast, decreased levels of sGC have been implicated in the pathogenesis of pulmonary hypertension in neonates. For example, Tzao et al. (47) found lower sGC expression and activity in the pulmonary arteries from lambs with ductal ligation-induced pulmonary hypertension than in controls. Alterations in NO sensitivity due to relatively low sGC protein levels may underlie the poor responsiveness seen to inhaled NO in some patients. Indeed, Bland et al. (10) found that lambs born prematurely who developed pulmonary hypertension had lower levels of sGC protein and activity than did age-matched controls. In addition, these lambs with pulmonary hypertension demonstrated attenuated responses to inhaled NO compared with age-matched controls. Furthermore, decreased sGC during inhaled NO therapy has been implicated in the pathogenesis of rebound pulmonary hypertension in a lamb model (44). Thus it appears that pulmonary expression of sGC is developmentally regulated and may contribute to changes seen in vasoreactivity to NO in the neonate.

A second potential mechanism for increased cGMP levels is lower activity or expression of PDE5 in the 14-day-old animals, which would result in a longer half-life of cGMP and thus higher intracellular concentrations of this second messenger. However we found no difference in lung PDE5 levels between the two age groups. In contrast, Hanson et al. (24) found that lung PDE5 levels and activity peaked at 7 days of age in neonatal mice and were lower at 14 days of age. Furthermore, Sanchez et al. (45) found that PDE5 activity is regulated during development. They showed cGMP PDE5 activity was greatest in 1-day-old pup lungs, intermediate in 8-day-old pup lungs, and least in adult rat lungs. Although alterations in PDE5 levels have been implicated in the pathogenesis of neonatal pulmonary hypertension, in the normal neonatal rat lungs examined in the present study, changes in PDE5 protein levels were not likely involved in the observed increased pulmonary vascular sensitivity to NO.

We found that the pulmonary vasculature of 7-day pups preconstricted with K+ responded to the cGMP analog 8-pCPT-cGMP to a greater degree than the preconstricted pulmonary vasculature of the 14-day-old pups. This suggests that the downstream mechanisms resulting in NO/cGMP-mediated vasodilation are intact in the lungs of 7-day pups but not accessible through NO stimulation. There may exist a disruption in NO-mediated signaling upstream of cGMP. As discussed above, this disruption may be related to decreased lung sGC protein levels, decreased sGC activity, or possibly increased lung levels of PDE5 protein and/or activity.

In summary, we found that basal production of NO is a major contributor to the normally low PVR in the neonatal lung. Furthermore, NO production increases between 7 and 14 days of age in neonatal rats. Downstream of NO production, expression of sGC is also greater at 14 days than at 7 days. The overall effect of these findings is that the pulmonary vascular bed depends more on the NO-cGMP pathway to regulate pulmonary vascular tone at 14 days than at 7 days of age. This supports our hypothesis that NO-dependent regulation of pulmonary vascular tone changes during early postnatal development. These findings may have important implications in the pathogenesis of postnatally acquired pulmonary hypertension in the neonatal period.

GRANTS

This work was supported by National Institutes of Health Grants HL-04050 (L. G. Chicoine), HL-58124 (B. R. Walker), RR-16480 and HL-77876 (T. C. Resta), and HL-075261 (L. D. Nelin).

REFERENCES


24. Jernigan NL, Walker BR, Resta TC. Regulation of bovine endothelial constitutive nitric oxide synthase by 10.220.33.1 on November 6, 2017 http://ajplung.physiology.org/ Downloaded from


