Relative contributions of endothelial, inducible, and neuronal NOS to tone in the murine pulmonary circulation

KAREN A. FAGAN, ROBERT C. TYLER, KOICHI SATO, BRIAN W. FOUTY, KENNETH G. MORRIS, J. R., PAUL L. HUANG, IVAN F. McMURTRY, AND DAVID M. RODMAN

Cardiovascular Pulmonary Research Laboratory and Department of Physiology, University of Colorado Health Sciences Center, Denver, Colorado 80262; and Harvard Medical School, Boston, Massachusetts 02114

Fagan, Karen A., Robert C. Tyler, Koichi Sato, Brian W. Fouty, Kenneth G. Morris, Jr., Paul L. Huang, Ivan F. McMurry, and David M. Rodman. Relative contributions of endothelial, inducible, and neuronal NOS to tone in the murine pulmonary circulation. Am. J. Physiol. 277 (Lung Cell. Mol. Physiol. 21): L472–L478, 1999.—Nitric oxide plays an important role in modulating pulmonary vascular tone. All three isoforms of nitric oxide synthase (NOS), neuronal (nNOS, NOS I), inducible (iNOS, NOS II), and endothelial (eNOS, NOS III), are expressed in the lung. Recent reports have suggested an important role for eNOS in the modulation of pulmonary vascular tone chronically; however, the relative contribution of the three isoforms to acute modulation of pulmonary vascular tone is uncertain. We therefore tested the effect of targeted disruption of each isoform on pulmonary vascular reactivity in transgenic mice. Isolated perfused mouse lungs were used to evaluate the effect of selective loss of pulmonary nNOS, iNOS, and eNOS with respect to hypoxic pulmonary vasoconstriction (HPV) and endothelium-dependent and -independent vasodilation. eNOS null mice had augmented HPV (225 ± 65% control, P < 0.02, mean ± SE) and absent endothelium-dependent vasodilation, whereas endothelium-independent vasodilation was preserved. HPV was minimally elevated in iNOS null mice and normal in nNOS null mice. Both nNOS and iNOS null mice had normal endothelium-dependent vasodilation. In wild-type lungs, non-selective NOS inhibition doubled HPV, whereas selective iNOS inhibition had no detectable effect. In intact, lightly sedated mice, right ventricular systolic pressure was elevated in eNOS-deficient (42.3 ± 1.2 mmHg, P < 0.001) and, to a lesser extent, in iNOS-deficient (37.2 ± 0.8 mmHg, P < 0.001) mice, whereas it was normal in nNOS-deficient mice (30.9 ± 0.7 mmHg, P = not significant) compared with wild-type controls (31.3 ± 0.7 mmHg). We conclude that in the normal murine pulmonary circulation 1) nNOS does not modulate tone, 2) eNOS-derived nitric oxide is the principle mediator of endothelium-dependent vasodilation in the pulmonary circulation, and 3) both eNOS and iNOS play a role in modulating basal tone chronically.

NITRIC OXIDE (NO) has been demonstrated to play a central role in modulating pulmonary vascular tone. This conclusion is supported by experiments in a variety of species, including humans, and from a variety of experimental systems, including isolated cells, arteries, isolated perfused lungs, and intact animals (2, 5, 9, 19, 24). Three isoforms of the enzyme responsible for NO production, nitric oxide synthase (NOS), have been described: neuronal (nNOS, NOS I), inducible (iNOS, NOS II), and endothelial (eNOS, NOS III). The principal isoform expressed in the normal pulmonary vasculature is eNOS (22, 23). However, the other two isoforms of NOS are also expressed in the lung, and expression of all three isoforms is reported to be increased in chronic hypoxic pulmonary hypertension (13, 23). iNOS is expressed constitutively in airway epithelium and can be detected by sensitive molecular techniques in airway and vascular smooth muscle as well (3, 27). nNOS is expressed in nonadrenergic noncholinergic nerve endings and bronchial epithelium, contributes significantly to lower airway NO production, and has been documented to play a role in regulating systemic vascular tone (4, 20, 29). Thus all three isoforms of NOS could potentially contribute to modulation of pulmonary vascular tone.

The relative contribution of the three isoforms of NOS to modulation of pulmonary vascular tone is uncertain. This uncertainty results largely from a lack of highly selective inhibitors of each isoform and the confounding effects of NOS inhibition on cardiac function and systemic vascular tone in vivo. Although several recent reports have suggested an important role for eNOS in the development of chronic pulmonary hypertension, the contribution of the individual NOS isoforms to acute modulation of pulmonary vascular tone has not been established (6, 25, 26). The recent construction of targeted deletions of each NOS isoform in transgenic mice provides a novel technique for testing the physiological role of the individual isoforms. To utilize these mice, we adapted the isolated, buffer-perfused lung preparation to the mouse lung, enabling us to monitor pulmonary vascular resistance during stimulation of the pulmonary circulation by hypoxia and pharmacological agents. We compared the effect of hypoxia, pharmacological NOS inhibition, and endothelium-dependent and -independent vasodilation in lungs from mice deleted selectively for nNOS, iNOS, or eNOS. We also measured right ventricular (RV) systolic pressure in lightly sedated mice as an estimate of pulmonary artery pressure in vivo.

MATERIALS AND METHODS

Transgenic Mice

Homozygous eNOS (eNOS −/−), nNOS (nNOS −/−), and iNOS (iNOS −/−)-deficient mice generated as previously

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described were used (10, 11, 16). Strain-specific, age-matched SV129-BL/6 controls were bred as the F1 generation of the cross of the parental wild-type strains C57BL/6 and SV129 (Jackson Laboratories and Taconic Laboratories, respectively). Animals were fed standard breeding rodent chow and water ad libitum and were housed under ambient conditions in the animal care facility located in Denver, CO.

RT-PCR

Whole lungs from mice were removed and immediately frozen in liquid nitrogen. Lungs were then minced and homogenized on ice in 5 volumes of TriReagent (Molecular Research Center). Total RNA was purified by phenol-chloroform extraction and precipitated with ethanol. RNA quality was assessed by gel electrophoresis. First-strand DNA synthesis was carried out with 5 µg of total RNA in a 20-µl reaction using Superscript II reverse transcriptase ( Gibco BRL). cDNA product (2 µl) was used as template for PCR to amplify eNOS, iNOS, and nNOS sequences. β-Actin primers were used as controls. Previously reported primer sequences and reaction conditions were used to amplify the sequences in mice (8). PCR products were evaluated on ethidium bromide agarose gel for determination of size and specificity.

Hemodynamic Studies

Isolated perfused lungs. Mice were anesthetized with phenobarbital (6–7 mg ip). After confirmation of deep anesthesia, thoracotomy was performed, and 100 units of heparin (Elkins-Sinn) were injected into the right ventricle. The thoraec was cannulated with an 18-gauge blunt-tipped catheter and was sutured in place, after which the mouse was ventilated with 21% O2, 5% CO2, and balance N2 at 60 liter and was sutured into place, after which the mouse was placed in a supine position while spontaneously breathing, and after calibration of the pressure transducer to the midanteroposterior diameter of the chest, a 26-gauge needle was introduced percutaneously into the thorax. Pulmonary pressure was measured using a pressure transducer (Statham), and real time tracings were obtained (Lockheed data recorder). The heart rate under these conditions was between 400 and 450 beats/min. If the heart rate fell below 300 beats/min, it was assumed that the level of anesthesia or trauma was inhibiting cardiac function, and those measurements were excluded from analysis. Approximately 1% of the animals was excluded for this reason. RV pressure was obtained successfully in all but two animals.

Determination of RV Hypertrophy and Hematocrit

After hemodynamic studies, the heart was removed en bloc from each animal and wrapped with saline-soaked gauze to prevent desiccation. The atria were then removed. The right ventricle was dissected from the left ventricle and septum, and each was weighed separately. RV-to-left ventricle and septum ratios were determined as an index of RV hypertrophy. Blood for hematocrit measurements were obtained by direct cardiac puncture in sedated mice immediately after percutaneous RV pressure measurements were made. Hematocrits were measured in capillary tubes using standard techniques.

Drugs and Chemicals

Unless otherwise stated in the text, all chemicals and reagents were obtained from Sigma.

Statistical Analysis

All results are presented as means ± SE. Comparisons were made using one- and two-way ANOVA with Fisher’s post hoc test, and P < 0.05 was accepted as significant.

RESULTS

We first performed RT-PCR analysis of mRNA for the three NOS isoforms in lungs removed from either wild-type or NOS-deleted mice. Examples of that experiment are shown in Fig. 1. mRNA for all three isoforms of NOS was detected in wild-type mouse lungs. RT-PCR also confirmed that targeted disruption selectively eliminated expression of the individual isoform of NOS in lungs from the transgenic mice.
We then questioned whether NO produced by the individual NOS isoforms modulated basal pulmonary vasoreactivity. To do so, we tested the effect of deletion of each NOS isoform on the magnitude of HPV in response to ventilation with 0% O₂. Figure 2 shows examples of acute HPV in wild-type and eNOS null lungs, demonstrating augmented HPV in the latter. Mean data are shown in Fig. 3; eNOS null mice demonstrated an approximate doubling of HPV compared with wild-type controls. Although iNOS null mice tended to have slightly greater HPV than wild-type controls, the difference was small and did not achieve statistical significance. HPV in nNOS null lungs did not differ from that in wild-type control lungs. The perfusion flow was maintained constant on a per weight basis throughout the experiments and resulted in a baseline perfusion pressure of 11.92 ± 0.41 mmHg in eNOS null lungs, 10.73 ± 1.1 in iNOS null lungs, 10.7 ± 0.33 in nNOS null lungs, and 9.3 ± 0.71 in wild-type lungs (P = not significant between all groups except P < 0.05 in eNOS null compared with wild type) despite the weights of the animals being different between the groups (31.4 ± 0.4 g in wild-type, 30.7 ± 1.7 g in eNOS null, 25.4 ± 1.6 g in iNOS null, and 21.3 ± 1.5 g in nNOS null, P < 0.05).

Next, we asked whether pharmacological inhibition of NOS augmented HPV in the isolated perfused lungs. Nonselective NOS inhibition by l-NNA doubled HPV in lungs from wild-type mice. However, l-NNA had little effect on HPV in iNOS null mice (Fig. 4). To further evaluate the role of iNOS-derived NO, we also tested the effect of the relatively iNOS-selective inhibitor aminoguanidine on HPV in wild-type lungs. Aminoguanidine had no effect on HPV compared with vehicle/time control (vehicle control 2.45 ± 1 mmHg vs. aminoguanidine 2.15 ± 1 mmHg, n = 4, P = not significant).

To evaluate the role of the NOS isoforms in endothelium-dependent vasodilation, we tested the effect of bradykinin on pulmonary vascular tone. Bradykinin was administered during the sustained phase of HPV. In all mice, an initial transient vasodilation was seen. In eNOS null mice, this transient vasodilation was followed within 1 min by sustained vasoconstriction. In contrast, in wild-type and nNOS and iNOS null mice, bradykinin vasodilation was maintained at ~50% of the maximum value. An example of bradykinin responses in wild-type and eNOS null lungs is shown in Fig. 5A. The average data from these experiments are shown in Fig. 5B. Sustained vasodilation was eliminated by pretreatment with l-NNA in wild-type lungs, confirming an obligatory role for NO in this response (data not shown).

**Fig. 1.** RT-PCR detection of mRNA for nitric oxide synthase (NOS) isoforms in wild-type (wt) and endothelial (e) NOS (e−/−), inducible (i) NOS (i−/−), and neuronal (n) NOS (n−/−)-deficient mice. Primers were detected by unique sequences in each isoform and produced fragments of 245, 338, and 404 bp, respectively, for eNOS, iNOS, and nNOS transcripts. mRNA for all three isoforms of NOS was detected in wild-type mouse lung. In contrast, the targeted deletions of NOS isoforms selectively eliminated mRNA for each individual isoform.

**Fig. 2.** Representative example of pressure tracings from isolated, buffer-perfused lungs from a wild-type and an eNOS null mouse. Three hypoxic challenges followed by addition of the nonselective NOS inhibitor Nω-nitro-L-arginine (l-NNA) and a final hypoxic challenge were performed. NOS inhibition did not increase perfusion pressure in either group. Subsequent hypoxic pulmonary vasoconstriction (HPV) was augmented in wild-type but not in eNOS null mice. PPa, mean pulmonary artery perfusion pressure.

**Fig. 3.** Group means from wild-type and the three NOS-deficient strains demonstrating that HPV was augmented in eNOS null mice and tended to be slightly increased in iNOS null mice, whereas it was normal in nNOS null mice. *P < 0.05 vs. wild type; n, no. of mice.

**Fig. 4.** Group means from wild-type and eNOS null mice demonstrating that nonselective NOS inhibition by l-NNA (100 mmol/l) augmented HPV in wild-type lungs and had only a small, statistically nonsignificant effect on eNOS null lungs. *P < 0.05 from wild type; n, no. of mice.
The failure of bradykinin to cause sustained vasodilation in eNOS null mice could have been due to defective NO production alone or also could involve inadequate smooth muscle cell guanylyl cyclase activity. We therefore tested the effect of the NO donor NONOate on pulmonary vascular tone during HPV. As shown in Fig. 6, vasodilation to NONOate was no different in wild-type and eNOS null lungs. Vasodilation to NONOate was also preserved in iNOS and nNOS −/− lungs.

To determine if in vitro pulmonary vascular reactivity measurements were relevant to in vivo physiology, we measured RV systolic pressure in lightly sedated mice exposed to chronic mild hypoxia (ambient conditions in Denver, CO, with inspired PO₂ ~122 mmHg). As shown in Fig. 7, eNOS null mice had elevated RV systolic pressure, whereas nNOS null mice had normal RV systolic pressure. iNOS null mice had a phenotype intermediate between control and eNOS null mice. Relative RV mass (RV-to-left ventricle + septum) ratio was 0.359 ± 0.06 in eNOS −/− mice (P < 0.05 compared with wild type), 0.232 ± 0.017 in iNOS −/− mice, 0.26 ± 0.02 in nNOS −/− mice, and 0.243 ± 0.01 in wild-type controls. Hematocrit did not differ between the four groups (0.444 ± 0.003 vs. 0.438 ± 0.012 vs. 0.435 ± 0.017 vs. 0.452 ± 0.003% in eNOS −/−, iNOS −/−, and wild-type mice, respectively).

DISCUSSION

Our studies tested the relative contributions of the three NOS isoforms to modulation of tone in the normal murine pulmonary circulation. RT-PCR confirmed that mRNA for all three isoforms was expressed in the mouse lung. This is consistent with findings in other species and suggested that all three isoforms could potentially contribute to control of pulmonary vascular tone. RT-PCR also confirmed that the targeted deletions had the anticipated effect on lung expression of the individual NOS isoform.

Physiological assessment in isolated perfused lungs demonstrated that HPV in eNOS-deficient lungs was approximately double that in wild-type lungs. In contrast, there was only a slight, nonsignificant increase in HPV in iNOS-deficient lungs, and HPV in nNOS-deficient lungs was equal to that seen in wild-type controls. These results suggest that, in the ex vivo perfused lung, eNOS is the principal source of the NO that modulates acute responses to hypoxia. Using the nonselective inhibitor of NOS L-NNA, we confirmed...
that the isolated mouse lung behaved similarly to that previously reported in the isolated rat lung in that nonselective NOS inhibition significantly augmented acute HPV. Consistent with eNOS being the predominant source of NO, L-NAN had only a minimal, statistically insignificant additional effect on HPV in eNOS null lungs. Also consistent with that hypothesis, the magnitude of HPV in wild-type lungs treated with L-NAN was double that of vehicle-treated lungs and no different from that seen in lungs from eNOS null mice.

Meng and co-workers (17) reported that nNOS compensates for the loss of eNOS in the brain of eNOS-deficient mice, resulting in preserved endothelium-dependent vasodilation. In contrast, Huang et al. (11) reported that isolated aortic ring segments from eNOS-deficient mice lacked ACh-mediated endothelium-dependent vasorelaxation, and Steudel et al. (25) observed paradoxical vasoconstriction to ACh in pulmonary artery rings after pharmacological inhibition of NOS in wild-type animals, although not in pulmonary artery rings from eNOS−/− mice. In vivo, Steudel and co-workers (25) found no evidence of endothelium-dependent vasodilation in response to ACh in the pulmonary circulation of eNOS-deficient mice. To clarify the role of the individual NOS isoforms in mediating pulmonary vascular endothelium-dependent vasodilation, we therefore tested the effect of bradykinin on tone in the isolated, perfused mouse lung. Like ACh used in other studies, bradykinin is an endothelium-dependent vasoconstrictor with a final common pathway of activation of eNOS (1). As expected, bradykinin caused prolonged pulmonary vasodilation during acute HPV in lungs from wild-type mice. Bradykinin pulmonary vasodilation in wild-type lungs was composed to two phases: a transient vasodilation that was unaffected by L-NNA, possibly due to endothelium-derived hyperpolarizing factor (18), and a sustained phase that was ~50% of maximum, inhibited by L-NNA, and therefore due to NO release. The transient phase was preserved in lungs from eNOS null mice. In contrast, the sustained phase was absent in eNOS null mice and was replaced by paradoxical vasoconstriction. Bradykinin-mediated vasodilation was normal in lungs from the other two NOS-deleted strains. Thus we find no evidence that either nNOS or iNOS participates in endothelium-dependent vasodilation in the mouse lung nor any evidence that these isoforms of NOS compensate for the loss of eNOS.

We found that endothelium-independent vasodilation to the NO donor NONOate was preserved in eNOS null mice after preconstriction with hypoxia. This contrasts with results of Steudel and co-workers (25), who found that the pulmonary circulation of eNOS null mice had impaired vasodilation to the NO donor sodium nitroprusside as well as inhaled NO in vivo but was intact in vitro when rings were preconstricted with 5-hydroxytryptamine. They concluded that there may be a defect in smooth muscle guanylyl cyclase (cGMP) activity in the pulmonary resistance bed of eNOS null mice in vivo or that NO had little effect on the resting pulmonary tone. Our results in the isolated perfused mouse lung do not confirm the first hypothesis and suggest that guanylyl cyclase function and other NO- and cGMP-stimulated pathways are preserved in the pulmonary resistance arteries of eNOS null mice. Additionally, we have recently reported that the response to inhaled NO in eNOS−/− mice remains intact, consistent with preserved downstream signal transduction in vivo (6).

In vivo measurements of RV systolic pressure were performed to determine if alterations in pulmonary vascular reactivity found in NOS-deficient lungs had physiological consequences in the intact animal. These in vivo measurements were obtained in mice that had been exposed to mild ambient hypoxia. The loss of eNOS resulted in a doubling of acute HPV in vitro and, as expected, increased RV systolic pressure in vivo, indicating the presence of pulmonary hypertension. This finding supports the role of eNOS in the development of chronic pulmonary hypertension. We have previously reported an increased sensitivity in eNOS-deficient mice to mild hypoxia (~33% increase) with respect to RV systolic pressure (compared with controls) that was attenuated by but not eliminated by conditions simulating sea level (~16% increase compared with controls; see Ref. 6). In vivo measurements of HPV in severe hypoxia were not obtained because the combination of general anesthesia and hypoxia resulted in significant cardiac compromise.

The loss of nNOS had no effect on acute HPV in vitro and no effect on RV systolic pressure in vivo. However, whereas the loss of iNOS and pharmacological iNOS inhibition had little effect on HPV in isolated lungs, iNOS null mice had an elevation in RV systolic pressure in vivo that was 56% of that seen in eNOS null mice. When compared with iNOS-deficient mice raised in sea-level conditions, the RV pressure in iNOS-deficient mice was increased by ~16% (data not shown). This disparity in the effect of iNOS disruption on HPV in isolated perfused lungs versus RV pressure in the intact animal suggests that an additional, extrapulmonary site of iNOS-mediated NO production contributes to modulation of pulmonary vascular tone in vivo.

iNOS has been found to be expressed constitutively in lower airway epithelium as a consequence of tonic cytokine stimulation (7). De Sanctis and co-workers (4) found that, in wild-type mice ventilated by tracheostomy, lower airway NO concentration was ~6 parts/billion. NO concentration was reduced to ~4 parts/billion in nNOS null mice, suggesting that while INOS may account for up to 60% of lower airway NO production, the total amount of NO produced in the lower airways is relatively small. We found a small, although not statistically significant, increase in HPV after NOS inhibition in eNOS null lungs. Thus it is possible that, in the isolated lung preparation in which the trachea was cannulated and ventilated, epithelial cell-derived NO from INOS provided a small contribution to control pulmonary vascular tone. Because our mice were kept in pathogen-free conditions, cytokine stimulation before death may have been minimal and thus airway iNOS may have been low relative to the situation in
normal animals and humans. However, if lower airways were the predominant site of iNOS-mediated NO production, we would have expected augmented HPV to be preserved in the isolated perfused lung preparation. Several studies from various species have demonstrated that the upper airway and sinuses are a significant source of NO in vivo. Kharatonov and co-workers (12) found that, in normal humans, NO concentration in the upper airway was ~1,000 parts/billion, whereas only 10 parts/billion were found in exhaled gas when the upper airway was occluded. The latter value is close to that observed in mice ventilated by tracheostomy and suggests that the upper airway could be an important source of NO. The relative contributions of iNOS, nNOS, and eNOS to production of upper airway NO have not been established in mice. However, Lundberg et al. (14) found that, in humans, paranasal sinus epithelium was the principal source of NO and identified iNOS as the predominant isoform of NOS using immunohistochemistry. Thus, in obligatory nose breathers such as mice, upper airway NO may provide a significant source of NO that is then delivered to the lower airways and pulmonary circulation during inspiration. Our finding that iNOS null mice have an intermediate degree of pulmonary hypertension when compared with eNOS null and wild-type mice is consistent with the hypothesis that iNOS-derived NO from the upper airway participates in chronic modulation of pulmonary vascular tone in vivo, although to a slightly lesser degree than does pulmonary vascular eNOS. The relevance of this mechanism of pulmonary vascular control to humans is supported by a recent preliminary report in postcardiac surgery patients demonstrating that nasal breathing resulted in significantly lower pulmonary vascular resistance than did oral breathing (15).

In conclusion, although all three isoforms of NOS are expressed in the mouse lung, it appears that only eNOS- and iNOS-derived NO modulate pulmonary vascular tone, whereas nNOS-derived NO does not. Our results are most consistent with a model in which the predominant isoform of NOS intrinsic to the pulmonary circulation is eNOS, whereas an extrapulmonary source of NO, most likely iNOS expressed in upper airway epithelium, also contributes substantially to modulation of pulmonary vascular tone in vivo.

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REFERENCES


