Contributions of nitric oxide synthase isozymes to exhaled nitric oxide and hypoxic pulmonary vasoconstriction in rabbit lungs

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NITRIC OXIDE (NO) produced within the lungs plays an important role in the regulation of a number of physiological and pathophysiological processes. These include bronchial and vascular smooth muscle relaxation, neurotransmission, and host defense. NO is produced by four isozymes of nitric oxide synthase (NOS): neuronal (nNOS or NOS I), inducible (iNOS or NOS II), and endothelial (eNOS or NOS III). Their expression and location in the respiratory tract vary between species and with disease states (reviewed in Ref. 13).

In mammals, exhaled NO is produced both by the epithelium of the nose and sinuses (15, 20) and the lower respiratory tract, including airways, blood vessels, nerves, and alveolar epithelium (6, 7, 25, 27–29). The sources and origins of exhaled NO from the lower respiratory tract remain poorly defined, both the anatomical location of its production and the relative roles of each isoform contributing to total NO synthesis. It has been claimed that the contribution of the vascular endothelium to exhaled NO is relatively minor in vivo as most of the produced NO is bound avidly and rapidly by hemoglobin in circulating red blood cells and surrounding vascular smooth muscle (27). However, we have demonstrated in certain situations that changes in vascular NO turnover can be reflected in exhaled NO (1).

The availability of selective NOS inhibitors and isoform-specific antibodies make it possible to study the relative contributions of the different isozymes to exhaled NO excretion, their location within the lung, their role in vascular regulation, and possibly the anatomical location of NO production appearing in exhaled gas. Thus we explored these questions in the isolated perfused rabbit lung using a variety of NOS inhibitors delivered by the vascular and inhaled routes and by immunocytochemistry. Studies were performed under normoxic and also under hypoxic conditions to determine the effects of inhibiting NOS isozymes on hypoxic pulmonary vasoconstriction (HPV), a critical ventilation-perfusion-matching mechanism known to be modulated by NO (7).

MATERIALS AND METHODS

Experimental preparation. The protocol was approved by the Animal Care Committee of the Veterans Administration Puget Sound Health Care System. Pathogen-free New Zealand White rabbits weighing 3.2 ± 0.2 kg, range 2.9–3.6 kg (West Oregon Rabbit Farm, Philomath, OR), were anesthetized with ketamine (10 mg/kg) and xylazine (0.33 mg/kg) injected through an ear vein. We performed a tracheotomy and ventilated the rabbit with a volume-controlled ventilator (Siemens Servo 900; Siemens) using a tidal volume of 10

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ml/kg, a respiratory rate of 20 breaths per min, zero positive end-expiratory pressure (PEEP), and inspired gas consisting of 21% O₂, 5% CO₂, and 74% N₂. Pancuronium (1 mg/kg) was administered at the onset of mechanical ventilation.

After a median sternotomy, the rabbits were anticoagulated with intravenous heparin at a dose of 300 U/kg. The right carotid artery was cannulated, and the rabbits were exsanguinated. The left atrium and main pulmonary artery were cannulated and perfused in an antegrade fashion with Krebs-Henseleit solution at a rate of 150 ml·kg⁻¹·min⁻¹ by means of a Masterflex pump (Cole-Palmer, Barrington, IL). Total circuit volume was 270 ml. The perfusate was warmed to 38°C with a countercurrent exchange heater.

The rabbit's chest was covered with plastic wrap to maintain 100% humidity and constant temperature. Pulmonary artery pressure (PAP) and left atrial pressure (LAP) were continuously measured, and LAP was kept constant at 5 mmHg by adjusting the height of the venous reservoir. Mixed exhaled NO was measured with a chemiluminescence detector (Sievers Instruments, Boulder, CO) by continuous sampling from a 50-ml reservoir placed in the expired gas line. The flow rate was 120 ml/min, out of a fixed ventilation of 600 ml/min. Calibration was performed with the normoxic and hypoxic ventilating gases passed through a NO scrubber to achieve a nominal zero (<1 ppb) and with a certified tank containing NO at 5.6 ppm (Air Liquide, Long Beach, CA). Increasing concentrations from 0 to 100 ppb were made by diluting volumes of calibrated NO stock gas with scrubbed gas.

After a 30-min stabilization period of normoxic ventilation, baseline measurements were taken. Then a 5-min hypoxic challenge was performed (5% O₂, 5% CO₂, 90% N₂). The lung was returned to normoxic ventilation for at least 5 min before any further new interventions (drug administration and hypoxic challenges) were undertaken. After each intervention, PAP, LAP, exhaled NO, and temperature were recorded. Serial blood gases were analyzed, and the pH was maintained between 7.35 and 7.40 with addition of sodium bicarbonate as necessary.

Pharmacological agents. N-[3-(aminomethyl)benzyl] acetamide (1400W) was used to selectively inhibit iNOS (34), and 7-nitroindazole (7-NI) was used to selectively inhibit nNOS (22). Both agents are active in the rabbit (23, 35), and the concentrations selected were those at the high end of sensitivity and specificity. At present there is no selective inhibitor of eNOS, thus its contribution was assessed indirectly by the use of nitro-l-arginine methyl ester (l-NNAME), a nonselective inhibitor of all isozymes (7, 17). The eNOS contribution was taken as the difference between the results obtained with 1400W plus 7-NI and those with l-NNAME. Acetylicholine (ACh) was infused in some preparations to increase NO production by the vascular endothelium (6, 14) to amplify the contribution of vascular NO to exhaled gas. Three groups were studied and are described below with their specific protocols.

Group A: effects of selective and nonselective NOS inhibitors on exhaled NO excretion and PAP in normoxia and hypoxia. After stabilization of the preparation, 7-NI and 1400W were added to the perfusate in a random manner. 1400W was diluted in 1.0 ml of normal saline and administered to yield a concentration of 0.4 μM. 7-NI was then administered to yield perfusate concentrations of 10 and 100 μM diluted in 1.0 ml of normal saline. These concentrations were chosen from previously published work (22, 34) in which near-maximal selective NOS isozyme inhibition was achieved. Fifteen minutes after the administration of each drug, PAP, LAP, exhaled NO, and airway pressures were recorded, and a 5-min period of hypoxic ventilation was initiated (n = 11). A 5-min period was then allowed before any further intervention. Controls received vehicle in the form of saline or saline with DMSO (n = 5).

Group B: effects of ACh infusion and NOS inhibitors on exhaled NO production and PAP in normoxia and hypoxia. To determine the effects of infused ACh and stimulated vascular endothelial NO production on exhaled NO excretion, we prepared the rabbit model as described above, and measurements were taken after 30 min. After this stabilization period, ACh dissolved in saline was infused at a dose of 1 μg·kg⁻¹·min⁻¹ (n = 5). Thereafter the protocol was identical to that of group A. In the control group, normal saline or saline plus DMSO was administered in place of all drugs (n = 6).

Group C: effect of nebulized l-NNAME on exhaled NO excretion and PAP in normoxia and hypoxia. To assess the airway and alveolar epithelial NOS contribution to exhaled NO and to determine vascular contributions, we administered l-NNAME by inhalation to concentrate its effect to nonvascular tissue. l-NNAME in increasing doses (dissolved in 1 ml of normal saline) was nebulized over a 5-min period (n = 5). Doses were based on final concentrations in the perfusate if all the l-NNAME was absorbed into the perfusate (100, 200, 500, 750, and 1,000 μM) with total doses of 7.4, 14.7, 36.9, 55.3, and 73.7 mg. A control group received nebulized saline in an identical manner (n = 4). At the end of each incremental dose of l-NNAME or saline, a 5-min hypoxic challenge was performed. In three experiments, l-NNAME concentrations in the perfusate following each nebulization were measured by ion exchange high-pressure liquid chromatography (Waters, Milford, MA) with a lithium buffer using postcolumn ninhydrin derivatization (31) (Pickering, Mountain View, CA).

Immunohistochemistry. Rabbit lungs were fixed in phosphate-buffered 4% formalin. Blocks of tissue from the middle and lower lobes of both the right and left lungs were dehydrated and embedded in paraffin. Sections were cut at 8 μm. Most of the immunohistochemical steps were performed in a “moist chamber” (a 150-mm plastic petri dish with a wet pad of filter paper). All reactions were at room temperature with the one exception noted below.

The three isozymes of NOS were detected with mouse monoclonal antibodies (anti-iNOS, catalogue no. N31020; anti-lNOS, catalogue no. N32020; anti-eNOS, catalogue no. N30020) from Transduction Laboratories (Lexington, KY). Secondary antibodies and reagents for peroxidase and diaminobenzidine staining were in a kit from Dako ARK (Animal Research Kit, Peroxidase; Carpinteria, CA). We performed negative controls by excluding the primary antibody during the staining procedure.

When it was obvious that we could not detect definite iNOS staining in the lung, we tested the antibody in a system known to express iNOS (a positive control) to assure its sensitivity and specificity. Murine macrophage cells (line J774A) were cultured in RPMI 1640 with 10% heat-inactivated fetal calf serum. For stimulation of iNOS, the cells were grown on glass coverslips in wells of 24-well plates until confluent. The medium was replaced with fresh medium containing various concentrations of Escherichia coli LPS (100, 50, 25, 12.5, and 6.25 μg/ml). After 48 h, the cells were fixed and processed for iNOS immunohistochemistry.

Antigen retrieval was done by flooding the sections with 1% SDS from Sigma (St. Louis, MO) for 5 min. Ten percent normal horse serum (Vector Laboratories, Burlingame, CA)
in Tris-buffered saline (TBS, from Dako) was used as a blocking buffer to suppress background staining (60 min at 37°C). Endogenous peroxidase activity was inactivated with 3% H2O2 for 5 min. After each of the steps, slides were washed with TBS.

Per the instructions in the Dako kit, the primary antibody was reacted with the biotinylated secondary antibody and blocking reagent before the antibodies were applied to the tissue sections. Primary antibody concentrations in the reaction mixtures were 5 μg/ml for anti-nNOS and anti-eNOS and 10 μg/ml for anti-iNOS. Reaction time for the antibodies on the sections was 15 min. Sections were treated with the streptavidin plus peroxidase reaction (15 min) and diaminobenzidine-chromagen staining (5 min). Sections were then rinsed in distilled water, lightly stained with Delafield’s hematoxylin, and coverslipped.

Statistical methods. All data are presented as means ± SD. Statistical significance was tested by paired t-test for within-group comparisons and were analyzed by the multiple analysis of variance followed by Scheffe’s and Bonferroni/Dunn post hoc tests corrected for multiple comparisons of similar data sets and for comparisons over time. A commercial software package (Statistical Package for the Social Sciences for Windows; SPSS, Chicago, IL) was used. Statistical significance was defined as P < 0.05.

RESULTS

Group A: effects of selective and nonselective NOS inhibition on exhaled NO excretion and PAP in normoxia and hypoxia. There was no significant decrease in exhaled NO following administration of 1400W and 7-NI compared with control or baseline levels of exhaled NO, but L-NAME (10 and 100 μM) caused a significant decrease (Fig. 1). There was no statistical change in exhaled NO in the control group at any point in the experiment. Baseline normoxic PAP did not change significantly following the administration of any inhibitor (Fig. 2). PAP rose slightly but insignificantly following hypoxic challenges in the control group (Fig. 3) and following 1400W or 7-NI. There was no difference in hypoxic PAP response between control and 1400W or 7-NI. With the administration of 10 μM L-NAME, however, there was a highly significant two-fold elevation in PAP following a hypoxic challenge (11 ± 11 mmHg vs. baseline, 1.5 ± 1.0 mmHg, P < 0.03). Several animals did not tolerate the hypoxic challenge following 100 μM L-NAME, developing unstable pulmonary hypertension and edema. Hence, this challenge was dropped, and these data are not reported.

Group B: effect of ACh and NOS inhibitors on exhaled NO production and PAP in normoxia and hypoxia. There was a small, statistically significant increase (P < 0.05, one-sided paired t-test) in exhaled NO with ACh infusion when the data from both the control and the experimental groups were pooled. Baseline NO was 58 ± 16 before and 63 ± 15 ppb after acetylcholine. Figure 4 shows no significant change in expired NO with administration of 1400W or 7-NI in this preparation. Exhaled NO fell markedly with administration of L-NAME at both concentrations (P = 0.001) compared with baseline. There was no effect of AcCh on normoxic PAP before or following administration of 1400W or 7-NI (Fig. 5). When the data from the control group and the experimental group were combined, there was also no change in the PAP in response to ACh. PAP rose, however, following administration of L-NAME (P = 0.01). After a hypoxic challenge (Fig. 6), there was no difference in the rise in PAP in the 1400W or 7-NI groups compared with the control group. However, there was a significant elevation in HPV following administration of L-NAME (Fig. 6; P = 0.01).
**Group C: effect of nebulized L-NAME on exhaled NO excretion and PAP in normoxia and hypoxia.** Figure 7 shows a steady progressive decrease in exhaled NO with increasing doses of nebulized L-NAME from 100 to 1,000 μM. Statistical significance ($P = 0.02$) began at 750 μM, whereas exhaled NO in the control group remained constant compared with baseline. Parallel to the fall in exhaled NO, Fig. 8 shows a progressive increase in the PAP change with hypoxia with significance beginning at 500 μM ($P = 0.02$). The PAP increase with hypoxia in the control group stayed relatively constant over the same time interval. Even after the highest dose nebulization of L-NAME, there was no detectable drug in the perfusate (<5 μM; the lower limit of detection of the amino acid analysis).

**NOS immunohistochemistry of rabbit lung.** Figure 9 shows the results of NOS isozyme immunohistochemistry in the lungs of normal healthy rabbits. By immunohistochemistry, there is definite cellular staining (tan to brown) for nNOS (NOS type I) and eNOS (NOS type III), with the latter showing greater staining intensity and more extensive cellular expression. Without primary antibody, no reaction product is found. These sections only show the blue hematoxylin staining.

nNOS is present in a moderate and uniform tan staining pattern in the ciliated cells of the bronchial epithelium, somewhat more intense than that of the bronchiolar epithelium. In addition, 40–80% of the epithelial nuclei are stained, the percentage increasing...
with the size of the bronchus. Mucous cells are not stained. Cartilage cells, both nuclei and cytoplasm, are markedly stained. A significant proportion of the cells in the bronchus-associated lymphoid tissue (BALT) is stained. The bronchiolar epithelium shows a uniform tan stain, intermediate in density between that of the bronchial epithelium and alveolar epithelium. Blood vessel walls and alveolar walls are only lightly stained.

iNOS (NOS type II) staining is essentially negative throughout the respiratory tract. The only definitive staining is in the ciliary layer of the bronchial epithelium. Of interest is the lack of iNOS staining in the immunological cells of the BALT. Because stimulated murine macrophage cells (line J774A), a cell line rich in iNOS, cultured with E. coli LPS, stained for iNOS, whereas control cells showed no iNOS staining (Fig. 10), the absence of iNOS in normal rabbit lungs is not a false negative finding.

Staining for eNOS was the most intense of the three NOS isoenzymes. Except for the mucous cells, bronchial epithelium shows intense brown staining. The cartilage cells stain strongly, but not the ground substance. Nearly all of the BALT cells are stained intensely, whereas the alveolar epithelium is moderately stained.
stained. Blood vessels show moderate staining. An apparent difference from nNOS staining is that eNOS staining is entirely, or nearly so, localized in the cytoplasm.

**DISCUSSION**

**NOS isozyme contributions to exhaled NO.** We have shown by using selective and nonselective NOS inhibitors that almost all exhaled NO in the isolated perfused rabbit lung is produced by eNOS and that only eNOS-derived NO modulates HPV. The extensive expression of eNOS by immunocytochemistry in the vascular endothelium as well as in the airway and alveolar epithelia, with very little nNOS and iNOS expression, further confirms this interpretation of the pharmacological data.

To our knowledge this work is the first to use isozyme-specific inhibitors to determine the isozyme origins of expired NO from the lower respiratory tract (airways distal to the larynx and lung parenchyma). DeSanctis et al. (8) measured differences in exhaled NO between tracheotomized normal mice and mice with a targeted deletion of nNOS and reported that nNOS is responsible for 40% of expired NO. The dif-
ference between our work and that of DeSanctis et al. (8) may represent either a species difference in lung NOS isozyme expression or an indirect effect of the gene deletion on other factors that control exhaled NO occurring in development that allows for survival to maturity without nNOS. Steudel et al. (32) studied total exhaled gas in awake, spontaneously breathing mice with targeted deletions of all three isozymes.

Fig. 8. Elevation in PAP following a hypoxic challenge in the group receiving nebulized L-NAME. The change in PAP during a hypoxic challenge rose steadily with nebulized L-NAME, achieving statistical significance beginning at 500 μM (P = 0.04).

Fig. 9. A panel of 20 micrographs illustrates the immunohistochemical results. The first column on the left shows tissue stained for neuronal NOS localization; the 2nd column shows inducible NOS (iNOS) localization; the 3rd column shows endothelial NOS localization; and the 4th column shows staining after the primary antibodies were not used. Tan/brown-stained areas show where NOS is localized at the histological level. Each row shows the histology of comparable areas as found in serial sections. The top row shows sections of bronchial epithelium and a portion of the underlying cartilage. The 2nd row again shows bronchial epithelium but of an area with underlying bronchial-associated lymphoid tissue. The 3rd row shows sections of bronchiolar epithelium. The 4th row shows sections of an arterial wall. The 4th row is of parenchyma and, on the right side of the micrographs, sections of venules. Magnification ×150. See RESULTS for further description.
surprising, in this study, which necessarily included NO produced by the nasal passages, there was no reduction of NO production with the iNOS deficiency and, interestingly, increased amounts of NO produced by the nNOS- and eNOS-deficient mice. There is no easy explanation for these results, but they point again to the complexity and difficulty in analysis of nonconditional gene knockout models as alluded to above.

NOS isozyme contributions to HPV. The absence of any difference in hypoxic PAP between control and intervention groups given 1400W and 7-NI strongly suggests that eNOS is the isozyme responsible for modulating HPV in the rabbit. Our NOS inhibitor results are in accord with the findings in NOS isozyme knockout mice, that eNOS is the principal isozyme whose NO production modulates both normoxic and hypoxic pulmonary tone (8, 11). Interestingly, although the absolute rise in PAP with hypoxia was greater with L-NAME without ACh pretreatment (group A), there was a larger increase in PAP in the ACh group (group B) in response to NOS inhibitors compared with respective control animals (Figs. 3 and 6). The hypoxic response of eNOS inhibition contrasted with the effect of L-NAME under normoxia. In the latter circumstance, although there was a large drop (>75%) in exhaled NO, there was no significant change in PAP, suggesting that normoxic baseline NO secretion is a minor factor in vasoregulation in the absence of hemoglobin.

One limitation of this study is that we employed a single concentration of 7-NI and 1400W. We chose the doses based on earlier studies that suggest activity of these NOS inhibitors at these concentrations (24, 35), although there were no previous studies used in studies of the lung. We cannot completely exclude some small effect of these isozymes, especially nNOS, if higher doses had been used. Similarly, we chose the dose of the ACh infusion based on earlier studies (6). There was a small increase in exhaled NO with ACh but no change in PAP. This contrasts with another study in whole rabbits that received intravenous nitroglycerin and had a dose-dependent rise in exhaled NO with a concomitant decrease in PAP (24). This rise in NO was unaffected by high-dose (30 mg/kg), intravenous L-NAME. A larger dose of ACh might have simultaneously increased exhaled NO and decreased PAP. Another possibility is that the pulmonary vasculature in this model may have been maximally dilated or nearly so at rest that the ACh would have little effect. These results then suggest that baseline NO levels under normoxia do not significantly affect pulmonary vasoregulation in the isolated, buffer-perfused rabbit lung.

Our study results are limited by the experimental model of the isolated, perfused rabbit lung. Thus our animals were perfused with nonpulsatile flow and were ventilated with zero PEEP. Carlin et al. (5) have shown that changing levels of PEEP change exhaled NO. When PEEP was increased, exhaled NO also increased. Also, these authors demonstrate decreased exhaled NO when flow was changed from nonpulsatile to pulsatile (5). These conditions remained unchanged during the study, but different ventilation or flow characteristics may have altered our data. Although our data and that of others (11, 12) suggest that vascular endothelium is important in regulating HPV, there is also an important contribution of airway eNOS-derived NO to pulmonary vascular regulation. It was our hypothesis in delivering L-NAME directly to the airways by nebulization that this route of administration would allow us to dissociate airway/alveolar NO production from vascular production. We reasoned that it might be possible to observe a reduction in exhaled NO before any augmentation of HPV occurred, a process generally considered to be dependent on a reduction of NO generated by the vasculature. However, the data in Figs. 7 and 8 clearly show that no such dissociation between HPV and exhaled NO change can be demonstrated. This intimate association of vascular responsiveness with airway and alveolar epithelial NO production is further supported by the lack of measurable L-NAME (~5 μM) in the perfusate even at the highest nebulized doses, a concentration well below the K_i for L-NAME against eNOS of ~100 μM (3, 26).

Thus it is most likely that nebulized L-NAME increased HPV by inhibiting airway and alveolar epithelial NO production and reducing the amount of NO.
available to diffuse from the airways to the vasculature. In support of this view, we have shown that dogs that exhaled NO may be reduced by three-quarters before any systemic hypertensive effect of nebulized L-NAME is measurable (2). Sartori et al. (27) drew similar conclusions in their analysis of the use of nebulized NOS inhibitors in humans. The caveat to this conclusion is that a small but sufficient amount of L-NAME may have diffused from the airways to bind to vascular eNOS and that complete scavenging by enzyme binding eliminated any drug available to accumulate in the perfusate.

Anatomic sources of expired NO. The anatomic sources of exhaled NO from the lower respiratory tract (airways, alveolar epithelium, and vessels) have been difficult to quantify not only for technical reasons but also owing to considerable species differences in the expression of and localization of the various NOS isozymes in the lung (reviewed in Ref. 13). There have been no NOS immunocytochemical studies of rabbit lungs reported heretofore.

eNOS. We found an extensive expression of eNOS in the airway epithelium (including the cilia), alveolar epithelium, and vascular endothelium of the rabbit, similar to the rat (37), but different from other mammals in which eNOS is not as strikingly expressed in the airways and alveoli. These findings are most consistent with a majority of exhaled NO arising from eNOS-derived NO from the airways and alveolar epithelium (1, 2, 4, 25, 27, 28). The rich expression of eNOS observed in the airways and alveolar epithelium of the rabbit may explain why this species has one of the highest concentrations of exhaled NO among mammals (2, 6, 7, 27).

nNOS. We found that nNOS is expressed in the bronchial epithelium in larger airways, specifically in the ciliated cells, but not mucous cells. This is consistent with findings in human lungs (16). The smaller airways show less intense nNOS staining. nNOS is present in lymphocytes associated with the BALT of the airways. Although nNOS is reported to be expressed in airway nerves of humans (16) and developing and adult mice (18), no definite staining of nerves was evident in any sections of rabbit airways we studied. As reported in human lungs (19), we found weak but definite staining in the capillary endothelium. Despite the presence of nNOS in the lung by immunohistochemistry, we could show only a nonstatistically significant small reduction in exhaled NO with 7-NI (Fig. 1). The reasons for the lack of drug effect may reflect the overwhelming dominant quantitative expression of eNOS in the rabbit lung, which makes detection of a small contribution difficult against a large background of eNOS-mediated production of NO.

iNOS. We could not detect iNOS in the airways or parenchyma of rabbits, except on cilia of the large airways. In fact, cilia in the large airways appear to contain all three isozymes. These findings are consistent with a role of local NO generation in rabbit airway ciliary motility (33) with upregulation with endotoxin challenge in rat lungs (10). Immunocytochemical evidence for all three isozymes has been reported for rat lungs (10, 37, 38). Although in humans and many species following inflammatory challenges (10, 13, 36) iNOS is readily detectable at the airway and alveolar levels, our studies were performed in pathogen-free rabbit lungs, so this may explain the lack of appreciable generalized airway and alveolar iNOS staining. The lack of effect of 1400 W on exhaled NO in the healthy rabbit lung parallels the absence of any quantitative contribution of the small amount of ciliary iNOS isozyme present in the airways.

The amount of NO produced by the vasculature that reaches exhaled gas is likely quite small but may be detectable under certain conditions. We found an increase in exhaled NO when ACh was administered in the perfusate. ACh is a known stimulant of vascular endothelial NO, and our result is similar to the ACh-mediated increase in exhaled NO observed in the isolated perfused pig lung (6). Although it is possible that ACh might have also increased epithelial NO production, studies in normal humans and asthmatics have shown no increase in exhaled NO with inhalation of methacholine, a related cholinergic agonist. (9, 30). We have also demonstrated a small vascular contribution to exhaled NO with large nonpharmacological increases in vascular pressure and flow in the saline-perfused rabbit lung (1). Given the relatively small increase in exhaled NO when vascular NO production is stimulated in blood-free perfused lungs, it seems reasonable to assume that in vivo very little NO produced by the vascular endothelium could escape the avid binding by red blood cell hemoglobin and reach airway and alveolar gas (7, 21).

In conclusion, we have shown pharmacologically and histochemically that, in the isolated perfused rabbit lung, most exhaled NO is produced by eNOS located in airways and alveolar epithelium and that this isozyme is involved in the modulation of HPV. A much smaller fraction of exhaled NO may derive from eNOS in the vascular endothelium. In the normal healthy rabbit, there is a small expression of nNOS in the airways that contributes minimally, if at all, to exhaled NO, and no expression of iNOS.

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