Research article

Upregulation of nitric oxide synthase in mice with severe hypoxia-induced pulmonary hypertension

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Abstract

Background: The importance of nitric oxide (NO) in hypoxic pulmonary hypertension has been demonstrated using nitric oxide synthase (NOS) knockout mice. In that model NO from endothelial NOS (eNOS) plays a central role in modulating pulmonary vascular tone and attenuating hypoxic pulmonary hypertension. However, the normal regulation of NOS expression in mice following hypoxia is uncertain. Because genetically engineered mice are often utilized in studies of NO, we conducted the present study to determine how hypoxia alters NOS expression in wild-type mice.

Method: Mice were exposed to sea level, ambient conditions (5280 feet) or severe altitude (17,000 feet) for 6 weeks from birth, and hemodynamics and lung NOS expression were assessed.

Results: Hypoxic mice developed severe pulmonary hypertension (right ventricular systolic pressure [RVsP] 60 mmHg) as compared with normoxic mice (27 mmHg). Using quantitative reverse-transcription PCR, it was found that expressions of eNOS and inducible NOS (iNOS) increased 1.5-fold and 3.5-fold, respectively, in the lung. In addition, the level of lung eNOS protein was increased, neuronal NOS (nNOS) protein was unchanged, and iNOS was below the limit of detection. Immunohistochemistry demonstrated no change in lung iNOS or nNOS staining in either central or peripheral areas, but suggested increased eNOS in the periphery following hypoxia.

Conclusion: In mice, hypoxia is associated with increases in lung eNOS, possibly in iNOS, but not in nNOS; this suggests that the pattern of lung NOS expression following hypoxia must be considered in studies using genetically engineered mice.

Keywords: hypoxia, mice, nitric oxide (NO), nitric oxide synthase (NOS), pulmonary hypertension

Introduction

NO, which may be synthesized by any of the three isoforms of NOS, is a vasodilator of the pulmonary circulation in many mammals. NO has been proposed as a modulator of vascular tone and structure in the pulmonary circulation, and previous studies using NOS inhibitors [1,2] suggested that inhibition of NO increases acute hypoxic pulmonary vasoconstriction. Chronic NOS inhibition did not lead to development of pulmonary hypertension [3], however, possibly because of a decrease in cardiac output. These dis-

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eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase; NO = nitric oxide; NOS = nitric oxide synthase; PBS = phosphate-buffered saline; PCR = polymerase chain reaction; RIPA = radioimmunoprecipitation assay; RVsP = right ventricular systolic pressure.

crepancies have been addressed in recent studies using mice that are deficient in NOS isoforms.

Three isoforms of NOS are found in the lung. The principle isoform that is found in the pulmonary vasculature is eNOS [4]. iNOS is expressed in airway epithelium, and airway and vascular smooth muscle [5,6], whereas nNOS is expressed in the bronchial epithelium and lung nervous tissue [4,5,7,8]. Thus, all three NOS isoforms could contribute to modulation of pulmonary vascular tone. Studies using knockout mice for each of these isoforms [9–12] suggest that eNOS-derived NO is important in modulating basal pulmonary vascular tone, as well as in attenuating the development of pulmonary hypertension.

Severe sustained hypoxia causes pulmonary hypertension in many animals. Upregulation of all three NOS isoforms following severe hypoxia has been reported in rats [4,13–16]. Less is certain about the expression of NOS in the murine lung following hypoxia, with previous reports [17,18] suggesting an increase in lung eNOS and iNOS levels. We therefore hypothesized that NOS isoforms are upregulated following hypoxia and that this may account for the increased susceptibility to hypoxic pulmonary hypertension in mice that lack eNOS [9,12]. In the present study we exposed wild-type mice to severe hypobaric hypoxia from birth and measured NOS mRNA and protein levels. We then compared the findings with localization of NOS protein in the lung as assessed using immunohistochemistry.

Materials and methods
Mice
We studied F1-generation SV129 (Taconic, Germantown, NY, USA) and C57BL/6 (Jackson Laboratories, Bar Harbor, ME, USA) mice at age 6 weeks.

Exposure environments
Within 24–36 h of birth, pups and dam were placed in ambient conditions, hypobaric hypoxia (simulating an altitude of 17,000 feet), or hyberbaric normoxia (simulating sea level). Exposure was continuous, with minimal interruption for animal care. At 21 days the dam was removed and animals were sorted by sex. At 6 weeks animals of both sexes were removed for study.

Right ventricular pressure measurements
RVsP was measured as previously described [9]. Briefly, mice were anesthetized with ketamine/xyalazine (100/15 mg/kg), placed supine while spontaneously breathing room air, and a 26-gauge needle was introduced percutaneously into the thorax via a subxyphloid approach; RVsP was recorded. Blood was drawn and animals were killed. The thorax was opened and the lungs were flushed with cold PBS. The right lung was frozen for protein and RNA measurement, and the left lung was fixed for histologic evaluation.

Western blotting
Frozen lung tissue was homogenized in ice cold 750 µl radioimmunoprecipitation assay (RIPA) buffer (1 × PBS, 1% Igepal CA-630, 0.5% sodium deoxycholate, 0.1% SDS) plus protease inhibitors (30 µl/ml RIPA aprotinin, 10 µl/ml RIPA 100 mmol/l sodium orthovanadate). Samples were triturated two to three times through 21-guage then 25-gauge needles, and 10 µl/ml RIPA 10 mg/ml phenyl methylsulfonyl fluoride was added. Samples were centrifuged twice at 4°C and 14,000 rpm. Protein concentration was determined using the Bradford method (Bio-Rad Laboratories, Hercules, CA, USA). Protein 10 µg (eNOS) or 80 µg (nNOS and iNOS) were separated by SDS-PAGE under reducing conditions (1% β-mercaptoethanol) using 8–16% gradient gels (Invitrogen, Carlsbad, CA, USA). Coomassie blue staining was used to verify equal loading of gel. Gels were transferred in 20% methanol to polyvinylidene difluoride membrane (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Blots were blocked (0.5% Tween 20, 1% milk) and incubated with primary antibody for 45 min at 37°C (1:1500 anti-eNOS mono [BD Transduction Laboratories, San Diego, CA, USA]; 1:1500 anti-nNOS poly [Zymed Laboratories, South San Francisco, CA, USA]; 1:200 anti-iNOS poly [Affinity Bioreagents, Golden, CO, USA]; and 1:1500 anti-β-actin mono [Sigma A5441; Sigma-Aldrich, St Louis, MO, USA]). Blots were washed and incubated with secondary antibody (1:1500 horseradish peroxidase) conjugated antimouse (eNOS) or antirabbit (nNOS, iNOS, and β-actin) for 45 min at 37°C. Blots were developed with Enhanced Chemiluminescence (Amersham Pharmacia Biotech). INOS-positive control was lipopolysaccharide-treated mouse spleen protein and nNOS-positive control was mouse brain protein.

Quantitative reverse-transcription polymerase chain reaction
Total RNA was extracted from 100 mg of frozen lung using Tri-reagent (Molecular Research Center, Inc., Cincinnati, OH, USA) and the final product was resuspended in 20 µl diethylpyro carbonate (depC) H_2O. RNA was quantified and samples with $A_{260}/A_{280} > 1.8$ were used. Samples were diluted in depC H_2O and 50 ng were used in quantitative one-tube reverse-transcription PCR (Applied Biosystems, Foster City, CA, USA). RNA quantity was determined using 18S rRNA primers/probes (Applied Biosystems) and eNOS, iNOS and nNOS expression determined using the primer/probe sequences shown in Table 1. Message abundance was determined by dividing the quantity of message of interest by the quantity of 18S ribosomal RNA.

Immunohistochemistry
Left lungs were inflated with 1% agarose, fixed in methyl carnoys (60% methanol, 30% chloroform and 10% glacial acetic acid), embedded in paraffin, and sectioned. Sections

Table 1

Primer/probe sequences used for determination of nitric oxide synthase isoform expression

NOS isoform	Primer/probe
eNOS	Forward: TCTGCGGCGATGTCACTATG
	Reverse: CATGCCGCCCTCTGTTG
	Probe: CCAGCGTCCTGCAAACCGTGC
iNOS	Forward: TGACGCCAAACATGACTTCAG
	Reverse: GCCATCGGGCATCTGGTA
	Probe: AATTCACAGCTCAATCCGGTACGCTGG
nNOS	Forward: GACTGATGGCAAGCATGACTTC
	Reverse: GCCCAAGGTAGAGCCATCTG
	Probe: TGGAACTCGCAGCTCATCCGCTATG

eNOS = endothelial nitric oxide synthase; iNOS = inducible nitric oxide synthase; nNOS = neuronal nitric oxide synthase; NOS = nitric oxide synthase.

were stained with anti-NOS antibodies (eNOS, BD Transduction Laboratories N30020, 1:400 dilution; iNOS, Zymed Laboratories 61-7000, 1:1000 dilution; nNOS, Transduction N32030, 1:500 dilution), developed with 3,3′-diaminobenzidine, and counterstained with hematoxylin.

**Hematocrit and nitric oxide metabolite measurements**
Heparinized blood 100 μl was placed in capillary tubes and hematocrit determined by standard techniques [9]. The remainder of the blood was spun at 4000 rpm for 10 min, and plasma removed and frozen. Later, samples were thawed and centrifuged at 15,000 rpm for 20 min. Plasma NO metabolites (NO, NO_2^-, NO_3^-, peroxynitrate, and nitrosothiols) were measured using a NO chemiluminescence analyzer (Ionics Instrument Business Group, Boulder, CO, USA). Plasma samples 1 μl were added to 2 ml vanadium III chloride (Sigma-Aldrich) dissolved in 1N HCl at 90°C to reduce all NO metabolites to NO. NO was driven into the chemiluminescence detector by bubbling with argon.

Statistical analysis
Data are expressed as mean ± standard error of the mean. Data were analyzed using analysis of variance, and $P < 0.05$ is considered statistically significant.

Results
Measurements of pulmonary hypertension
RVsP was measured as an index of pulmonary arterial pressure. As shown in Fig. 1, RVsP was elevated in animals that were exposed to hypobaric hypoxia as compared with those that were exposed to sea level and ambient conditions. Hypoxic animals were also more polycythemic than were normoxic animals (70% ± 4% in hypoxic animals versus 45% ± 3% and 42% ± 3% for

Figure 1
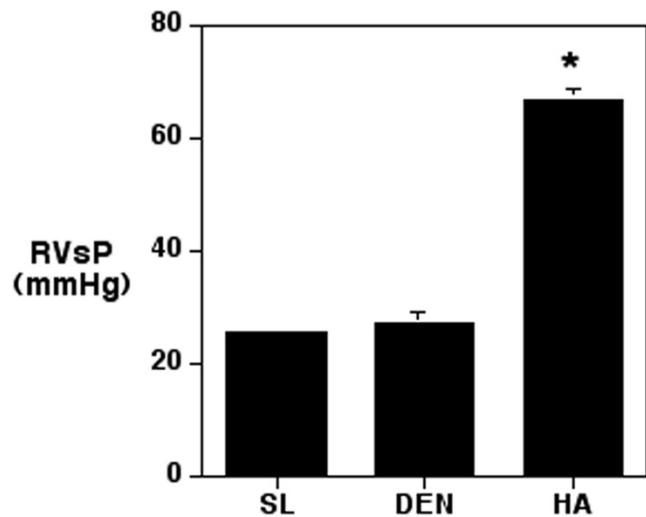
Right ventricular systolic pressure (RVsP) in mice exposed to sea level conditions (SL, $n = 5$), ambient conditions (DEN, $n = 9$), or hypobaric hypoxia (HA, $n = 6$) for 6 weeks after birth. *$P < 0.005$ versus SL or DEN.

animal subjected to sea level and ambient conditions, respectively; $P < 0.001$).
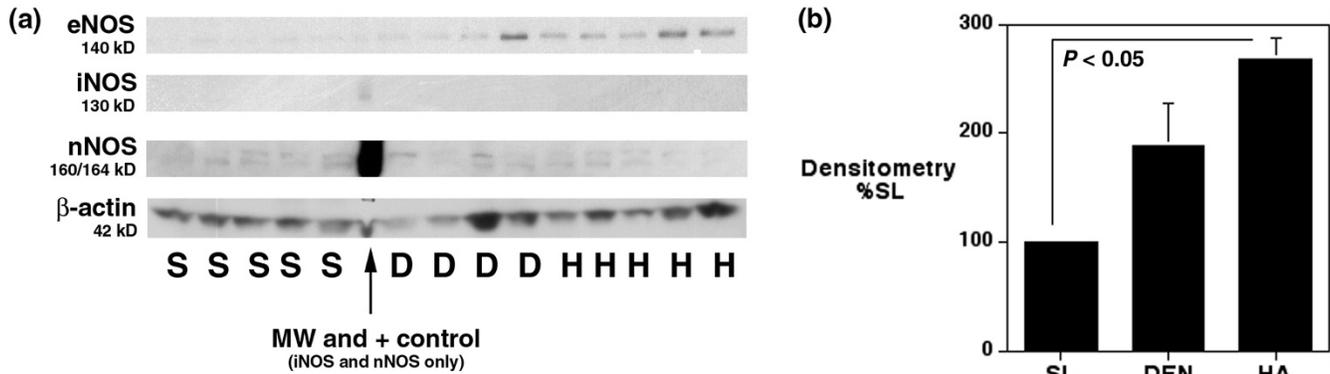
Western blotting
As shown in Fig. 2, lung eNOS protein levels were increased following hypoxia, with a trend toward increased eNOS in ambient versus sea level conditions. However, iNOS protein was below the limit of detection following any level of hypoxic exposure (positive control was lipopolysaccharide-treated mouse spleen) and using several different antibodies (Affinity Bioreagents and BD Transduction Laboratories; Affinity Bioreagents blot shown), and nNOS was unchanged among the three experimental groups (positive control was mouse brain).

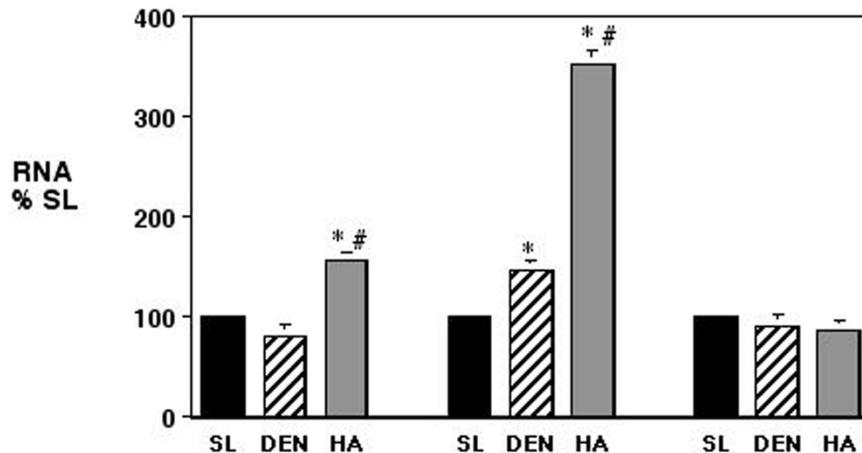**Quantitative reverse-transcription polymerase chain reaction**
eNOS expression in the lung, as studied using quantitative reverse-transcription PCR, was increased 1.5-fold following hypoxia as compared with normoxic conditions, whereas iNOS expression was increased 3.5-fold (Fig. 3).

Immunohistochemistry
Localization of NOS in mouse lung was determined using antibodies specific for eNOS, iNOS, and nNOS (Fig. 4). eNOS levels were increased following hypoxia, and the staining was more intense in large vessels and extended out to the periphery of the lung, and was also found in smaller vessels. In contrast, iNOS and nNOS were limited primarily to the airway epithelium, and their levels did not increase following exposure to hypoxia. This was confirmed using quantitative image analysis (data not shown).

Figure 2

Lung nitric oxide synthase (NOS) protein. **(a)** Western blots of homogenized whole lung from mice exposed to sea level conditions (S, $n = 5$), ambient conditions (D, $n = 4$), or hypobaric hypoxia (H, $n = 5$) for endothelial NOS (eNOS; 140 kDa), inducible NOS (iNOS; 130 kDa), neuronal NOS (nNOS; 164 and 160 kDa), and β-actin (42 kDa). Positive control is present for iNOS (lipopolysaccharide-treated mouse spleen protein) and nNOS (mouse brain protein). nNOS protein on Western analysis has previously been reported to appear as a doublet [45]. MW, molecular weight marker. **(b)** Densitometry evaluation for eNOS protein comparing sea level (SL, $n = 5$) with ambient conditions (DEN, $n = 3$) and hypobaric hypoxia (HA, $n = 5$). *$P < 0.05$ SL versus HA.

Figure 3

Quantitative reverse-transcription PCR for endothelial nitric oxide synthase (eNOS), inducible NOS (iNOS), and neuronal NOS (nNOS) in homogenized whole lungs from mice exposed to sea level conditions (SL, $n = 5$), ambient conditions (DEN, $n = 4$), or hypobaric hypoxia (HA, $n = 5$) for 6 weeks after birth. *$P < 0.05$ versus SL, #$P < 0.05$ versus DEN.

Nitric oxide metabolites

To determine whether the increase in NOS in the pulmonary circulation following hypoxia resulted in increased NO, we measured plasma NO metabolites (Fig. 5). There was a modest increase in levels of NO metabolites in animals exposed to hypoxia as compared with sea level or ambient conditions.

Discussion

The major finding of this work was the upregulation of lung expression of eNOS and possibly iNOS in mice with severe hypoxia-induced pulmonary hypertension. The increased eNOS was correlated with increased immuno-

localization to the periphery of the lung. This, however, was not observed for iNOS or nNOS. Additionally, we found increased severity of pulmonary hypertension in animals exposed to hypoxia during postnatal development.

The pulmonary hypertension in mice exposed to hypoxia from birth was more severe than we had observed in previous studies of 4 weeks of hypoxia in mature animals [9]. The degree of polycythemia was also greater in these animals than in previous reports, and might have contributed to the increase in pulmonary pressure due to increased viscosity [19]. This suggests that either the duration of hypoxic exposure or exposure from neonatal
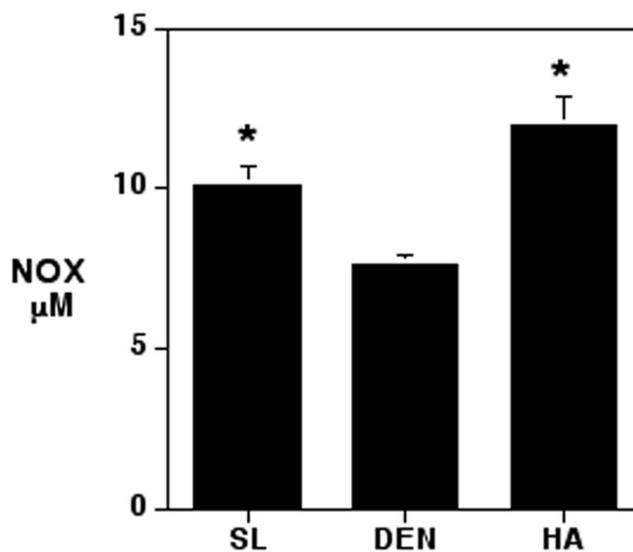
Figure 4
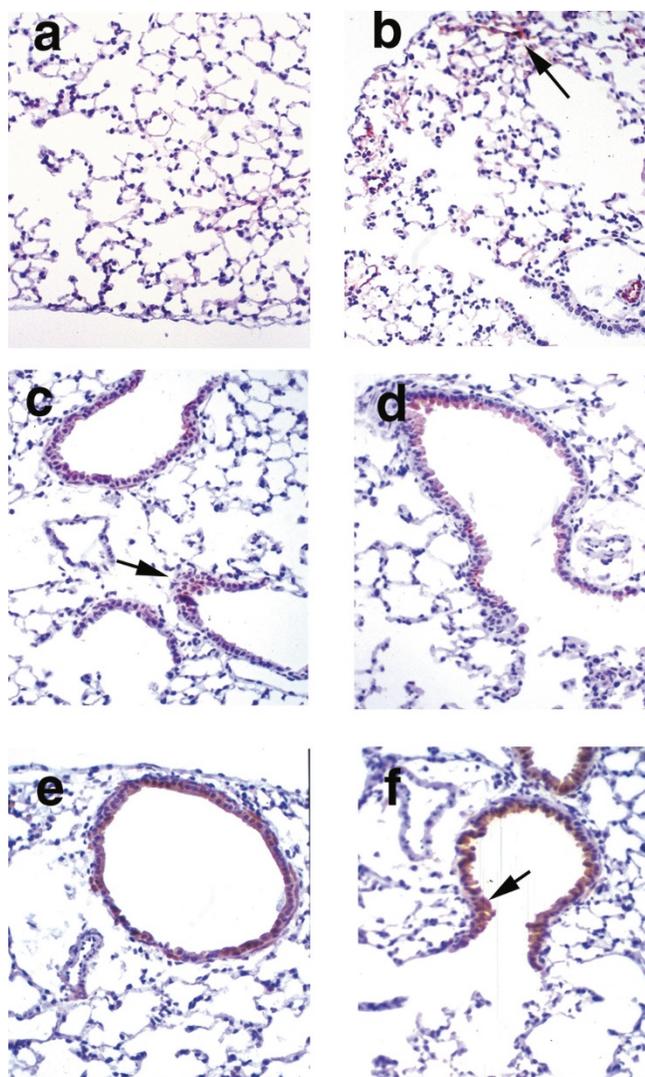
Lung nitric oxide synthase (NOS) immunolocalization. Lung endothelial NOS (eNOS) immunostaining in **(a)** normoxic versus **(b)** hypoxic mice, demonstrating an increase in peripheral distribution of eNOS (arrow, red staining). Lung inducible NOS (iNOS) immunostaining in **(c)** normoxic versus **(d)** hypobaric mice, demonstrating no difference in the airway epithelial localization of iNOS (arrow, brown staining). Lung neuronal NOS (nNOS) immunostaining in **(e)** normoxic versus **(f)** hypoxic mice, demonstrating no change in airway epithelial distribution of nNOS (arrow, brown staining). Magnification 40×.

Figure 5

Plasma nitric oxide metabolites (NOX) from mice exposed to sea level conditions (SL, $n = 5$), ambient conditions (DEN, $n = 9$), or hypobaric hypoxia (HA, $n = 6$) for 6 weeks after birth. *$P < 0.05$ versus DEN.

life provided additional physiologic stress that was not seen in more mature animals [9]. No animals died, however, suggesting that the stress was not sufficiently severe to decrease survival during the study period. This observation of increased susceptibility to hypoxic pulmonary hypertension in younger, developing animals is in agreement with previous reports [20,21].

The increase in eNOS protein and mRNA levels, and the peripheral immunolocalization is in accord with previous reports [13,14,18]. LeCras *et al*. [13] found that the amount of eNOS protein was increased in hypoxic, hypertensive rat lungs, and that this was not entirely due to the presence of hypertension and increased shear stress, but was also due to hypoxia. This is further supported by Tyler *et al.* [15], who found that eNOS protein levels were increased in rats with hypoxic pulmonary hypertension but not in those with chemical-induced (monocrotaline) or genetic (fawn-hooded rat) pulmonary hypertension [15]. As in the present study, increased eNOS levels correlated with *de novo* expression in the small resistance vessels in the lung [14,18]. The mechanism by which hypoxia increases eNOS expression is poorly understood because a hypoxia responsive element has not yet been identified in the eNOS promoter. Recently, altering the redox state of pulmonary artery endothelial cells in culture suggested that activation of redox-sensitive transcription factor activator protein-1 during hypoxia may be responsible for the increase in eNOS expression [22]. Despite the increase in eNOS, however, the mice developed severe pulmonary hypertension; this suggests either that NO production was not increased (see below) or that NO was consumed. Under more physiologically relevant levels of hypoxia, the central role of eNOS in protecting against hypertension is supported by our observation that loss of eNOS leads to increased acute hypoxic vasoconstriction and development of pulmonary hypertension [9–12].

In contrast to the observations with eNOS, iNOS message was increased but iNOS protein remained below the limit of detection in the lungs of hypoxic, hypertensive

mice. Previously, LeCras *et al.* [14] reported an increase in both iNOS mRNA and protein levels in rat lungs following hypoxia. In the present study, however, the increase in iNOS mRNA levels was not associated with detectable iNOS protein, using several different antibodies, in mice. This is in contrast to the study of Quinlan *et al.* [17], in which increased lung iNOS protein levels in adult mice following hypoxia was reported. This discrepancy may be due to differences between the present study and that report. Specifically, animals in our experiments are a different strain and were housed in sealed hypobaric chambers as opposed to being flushed with 10% O_2 gas. The hypobaric chambers used in the present study have a higher rate of air exchange than other hypoxic gas chambers, thus limiting the build-up of waste products, including ammonia, that may contribute to lung inflammation and induction of iNOS, as the combination of hypoxia and inflammation has previously been reported to increase iNOS expression [23]. Thus, the increased iNOS reported previously may be due to the combination of hypoxia and inflammation [23]. Others have also reported that lung iNOS protein was detectable only after lipopolysaccharide administration [24].

The iNOS promoter contains a hypoxia responsive element, and the increase in iNOS transcription in hypoxia may be due to the transcription factor hypoxia-inducible factor-1α [25–27]. Additionally, recent studies also suggest that alterations in redox state during hypoxia may also increase iNOS expression, similar to eNOS, by activation of redox sensitive nuclear factor-κB or activator protein-1 [28,29]. It is not known whether hypoxia interferes with translation or degradation of iNOS protein. In support of our current finding, however, immunohistochemical localization of iNOS to the airway did not change following hypoxia. We have recently reported that mice deficient in iNOS do not have increased pulmonary vasoreactivity, suggesting that lung-derived iNOS is not an important modulator of acute pulmonary vascular reactivity [10]. Mice deficient in iNOS do have a slight increase in right ventricular pressure, however, suggesting that iNOS, especially in the upper airway, may contribute to modulation of chronic pulmonary vascular tone [10]. Thus, the importance of iNOS in hypoxic pulmonary hypertension remains uncertain.

In rat lungs, nNOS expression was increased following hypoxia [4], but we did not find an increase in lung nNOS in mice following hypoxia. Increased nNOS has been suggested as a compensatory vasodilator in the cerebral and coronary circulation of eNOS-deficient mice, but its role in the pulmonary circulation is unknown [8,30]. However, we previously demonstrated [10] that nNOS does not appear to play a role in modulating tone in the pulmonary circulation.

Although we did not look at the expression of NOS in other rat vascular beds following hypoxia, other reports suggest that the increase in NOS following hypoxia may be species and tissue specific. Previous studies of NOS expression in systemic and pulmonary endothelial cells from different species in cultures [31–37] have yielded conflicting results. Recently, Toporsian *et al.* [38] reported *in vivo* downregulation of eNOS mRNA and protein levels in the aorta of rats following prolonged hypoxia, which correlated with impaired endothelium-dependent vasorelaxation, whereas there was an increase in eNOS protein levels in the lungs. Conversely, eNOS levels were increased in hypoxic uterine, but not femoral or renal arteries of pregnant sheep [39].

Whether increased NOS expression leads to increased production of NO *in vivo* is controversial [40]. In piglets *in vivo*, acute hypoxia caused decreased exhaled NO and decreased aortic and pulmonary arterial plasma nitrite levels [41]. Following chronic hypoxia in rats, Sato *et al.* [42] found that plasma NO metabolite levels were markedly increased compared with normoxic controls when animals were exposed to 21% O_2, but were not increased when rats were breathing 10% O_2. This suggests that hypoxia inhibited NO production despite the increase in NOS expression. There are several proposed mechanisms for the discordance between NO production and increased NOS expression *in vitro* and *in vivo*, including impaired arginine uptake [43], decreased heat shock protein-90 levels [44], and decreased O_2 tension [42,40]. In agreement with previous studies, the plasma NO metabolite content in the present study was minimally elevated in the hypoxic group. Although we attempted to keep the mice continuously hypoxic, a relatively brief period of reoxygenation might account for the slight increase in plasma NO metabolites seen in the hypoxic animals, which is consistent with the observations of Sato *et al.* [42]. Because the systemic and pulmonary vasculatures can both contribute to plasma NO metabolite levels, if NOS was downregulated in the systemic circulation as suggested by Toporsian *et al.* [38], then the lack of increased NO metabolites could also be due to decreased NOS activity in the systemic circulation. However, those investigators did not observe any difference in plasma NO metabolites in normoxic versus hypoxic rats in which aortic NOS was downregulated, but did observe a decrease in acetylcholine-stimulated NO release from aortic rings. The pattern of NOS expression in vascular beds other than the lung following hypoxia in mice is presently unknown.

Conclusion

In summary, we identified a unique pattern of NOS upregulation in the murine lung with severe hypoxia-induced pulmonary hypertension, which may be important when considering studies using NOS-deficient mice. The

present study also suggests that exposure of neonatal mice to hypoxia may lead to more severe pulmonary hypertension than that observed in mature mice exposed to hypoxia, and this warrants further study.

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