eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia

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Quinlan, Timothy R., Dechun Li, Victor E. Laubach, Edward G. Shesely, Nan Zhou, and Roger A. Johns. eNOS-deficient mice show reduced pulmonary vascular proliferation and remodeling to chronic hypoxia. Am J Physiol Lung Cell Mol Physiol 279: L641–L650, 2000.—Pulmonary hypertension is characterized by structural and morphological changes to the lung vasculature. To determine the potential role of nitric oxide in the vascular remodeling induced by hypoxia, we exposed wild-type [WT(+/+)] and endothelial nitric oxide synthase (eNOS)-deficient [(-/-)] mice to normoxia or hypoxia (10% O2) for 2, 4, and 6 days or for 3 wk. Smooth muscle α-actin and von Willebrand factor immunohistochemistry revealed significantly less muscularization of small vessels in hypoxic eNOS(-/-) mouse lungs than in WT(+/-) mouse lungs at early time points, a finding that correlated with decreases in proliferating vascular cells (5-bromo-2`-deoxyuridine positive) at 4 and 6 days of hypoxia in the eNOS(-/-) mice. After 3 wk of hypoxia, both mouse types exhibited similar percentages of muscularized small vessels; however, only the WT(+/-) mice exhibited an increase in the percentage of fully muscularized vessels and increased vessel wall thickness. eNOS protein expression was increased in hypoxic WT(+/-) mouse lung homogenates at all time points examined, with significantly increased percentages of small vessels expressing eNOS protein after 3 wk. These results indicate that eNOS deficiency causes decreased muscularization of small pulmonary vessels in hypoxia, likely attributable to the decrease in vascular cell proliferation observed in these mice.

Nitric oxide (NO) is a highly reactive gas that produces a variety of physiological and pathophysiological effects (26, 28). NO is an important modulator of blood pressure due to its vasodilatory capabilities as an endothelial cell-derived relaxing factor (EDRF) (11, 26, 33). In addition, many studies (2, 8, 9, 29) have demonstrated that NO is involved in modulation of cell proliferation, differentiation, and apoptosis in the vasculature. NO has been shown to exert both proliferative and antiproliferative effects on vascular SM (1, 6, 23, 29, 35, 46). The site of origin of NO, its concentration, and the stage of remodeling are all factors that may influence which action NO shows.

Many studies (7, 18, 39, 40, 45) have led to conflicting data about the roles of NO in the development of the structural and pathological changes seen in the vasculature of hypoxia-induced pulmonary hypertensive lungs. Although some studies (18, 45) have implicated alterations in the NO signaling pathway in the pathogenesis of pulmonary hypertension, others (12, 13, 20) have yielded conflicting results that indicate a protective and antiproliferative role for NO in the pulmonary vasculature. In vivo experiments performed in our laboratory revealed an upregulation of both the constitutive endothelial (eNOS) and inducible (iNOS) isoforms of NO synthase (NOS) and NO production in the lung vasculature of rats exposed to chronic hypoxia (18, 45). Elevations in NOS expression correlated temporally with the onset and progression of remodeling of these vessels (45). Meanwhile, the mRNA, protein, and enzyme activities of the NO receptor protein-soluble guanylate cyclase are all increased in rat lungs exposed to chronic hypoxia (21). Recent studies that used mice with homozygous-targeted disruptions of the eNOS gene [eNOS deficient (-/-)] have yielded insight into the potential role of endothelium-derived NO in the etiology of pulmonary hypertension and vessel re-

VASCULAR REMODELING, a dynamic process that occurs in response to a variety of stimuli, including shear stress and hypoxia, is one of the characteristics of pulmonary hypertension. The remodeling process of the pulmonary vasculature in response to hypoxia-induced pulmonary hypertension is known to involve structural alterations that extend to the periphery of the vascular tree and includes thickening of the smooth muscle (SM) layer down to the precapillary vessels (15). This muscularization of existing vessels involves both hy-
modeling. eNOS(−/−) mice exhibit both systemic and pulmonary hypertension. However, in eNOS(−/−) mice, there is increased pulmonary vascular remodeling in hypoxia-induced pulmonary hypertension (7, 37, 39).

In the studies presented here, we attempted to elucidate the roles of endothelium-derived NO in hypoxia-induced pulmonary vascular remodeling and, in particular, during the early phase of remodeling when hyperplasia and vascular cell proliferation are most prominent. To accomplish this, we exposed both wild-type [WT(+/+)] and eNOS(−/−) mice to a time course of normoxia or chronic hypoxia. We then examined the pulmonary vasculature of these mice to determine the kinetics of their remodeling response to hypoxia. In addition, we examined whether proliferation of vascular cells plays a mechanistic role in this remodeling and what effect the deletion of eNOS produces on proliferative indexes and the vascular remodeling response to hypoxia. We found that remodeling of the small pulmonary vessels begins as early as 2 days of hypoxia, with cell proliferation in the pulmonary vasculature distinctly upregulated by 4 days of exposure. In contrast to previous reports (7, 40), we observed that remodeling of the small pulmonary vessels after exposure to chronic hypoxia compared with that in WT(+/+) mice. Furthermore, the underlying mechanism for this remodeling deficit is a decrease in vascular cell proliferation in the small vessels of the eNOS(−/−) mice compared with that in the WT(+/+) mice.

MATERIALS AND METHODS

**Animal exposures.** WT(+/+) C57BL/6J (Jackson Laboratories, Bar Harbor, ME) or eNOS(−/−) mice aged 8–12 wk (n = 5–8 total/group) were generated as previously described (20) and backcrossed at least seven generations to the parental C57BL/6J strain. The mice were exposed to either normoxia or hypoxia (10% O2) for 2, 4, and 6 days or for 3 wk according to published methods (18, 21, 42). Forty-eight and twenty-four hours before tissue harvest, all animals were injected intraperitoneally with 100 mg/kg body wt of 5-bromo-2′-deoxyuridine (BrdU; Boehringer Mannheim, Indianapolis, IN). All animals were fed standard mouse chow and water ad libitum and were treated humanely in accordance with institutional and federal guidelines.

**Tissue processing.** At the indicated time points, each mouse was anesthetized with methoxyflurane, the chest cavity was opened, and the lungs were perfused with 2–4 ml of heparinized saline through the pulmonary artery until they appeared white. The lungs were removed and slightly inflated through the trachea with Ca2+- and Mg2+-free PBS (GIBCO BRL, Life Technologies, Grand Island, NY). The left lobe was sectioned into 1- to 2-mm sections, fixed in 4% paraformaldehyde in PBS, and processed for paraffin-embedded and frozen sections as previously described (45). The right lung lobe was flash-frozen in liquid nitrogen and stored at −70°C for Western blot analysis.

**Immunohistochemical techniques: muscularization of pulmonary vessels.** Frozen sections (3–5 μm) were immunolabeled with antibodies specific for von Willebrand factor (vWF) for endothelium and SM α-actin for SM cells to differentiate these tissue types, as previously described (45). In brief, tissue sections were incubated overnight at 4°C with a rabbit polyclonal and a monoclonal antibody for vWF (DAKO, Carpinteria, CA) and SM α-actin (St. Louis, MO), respectively. The sections were subsequently incubated with peroxidase-conjugated goat anti-rabbit (Bio-Rad, Hercules, CA) and alkaline phosphatase-labeled rabbit antimouse (Sigma) secondary antibodies. vWF staining was visualized with the 3′-diaminobenzidine (Vector, Burlingame, CA) substrate, which produces a brown/black color. SM α-actin staining was accomplished with the New Fuchsin (Dako) substrate, which produces a red color. Sections were then briefly counterstained with hematoxylin before they were mounted.

**BrdU incorporation.** BrdU is a nonradioactive thymidine analog incorporated into the DNA of actively proliferating cells only. Immunohistochemical staining of paraffin sections was performed as follows. Sections (3- to 5-μm) were baked onto slides at 60°C. Lung tissue was then digested with 0.1% trypsin (Sigma) before denaturation of DNA with 4 M hydrochloric acid to expose the antigen. After neutralization of the acid with PBS, the sections were incubated overnight at 4°C with an alkaline phosphatase-labeled monoclonal anti-BrdU antibody (Boehringer Mannheim). Identical procedures for the vWF immunolabeling mentioned in Immunohistochemical techniques: muscularization of pulmonary vessels were performed the next day. BrdU localization within proliferating cells was visualized with Vector Blue substrate (Vector Laboratories), and sections were counterstained with Nuclear Fast Red (Vector Laboratories) before they were mounted.

**Vessel morphometry and cell proliferation.** All analyses of vessel morphometry and cell proliferation were performed with the use of a blind code. The degree of muscularization of small (≤80 μm), medium (≥81–149 μm), and large (>150 μm) vessels was determined in a minimum of 200 small, 50 medium, and 25 large vessels per animal. Vessels were classified as nonmuscular, partly muscular, or fully muscular by SM α-actin staining. Partially muscular vessels were defined as those exhibiting at least one SM cell but no continuous media. Fully muscularized vessels had a continuous SM layer. The number of vessels exhibiting at least one SM cell divided by the total number of vessels yielded the percentage of muscularized vessels. Muscularized vessels were then classified as partly or fully muscularized by the criteria described above. The mean of repeated measurements from the endothelium to the outer edge of the SM layer in fully muscularized vessels was used in the formula (2 × wall thickness × 100)/external diameter to give percent vessel wall thickness (%VWT) or, for the three sizes of vessels, to analyze increases in VWT vascular remodeling.

Two parameters were measured to assess cell proliferation via BrdU incorporation into the pulmonary vasculature. The percentage of total vessels with BrdU-positive cells was evaluated in a manner identical to the one outlined above, and the number of BrdU-positive cells per positive vessel was determined. In vessels with either an incomplete SM layer or no SM layer, only BrdU-positive cells immediately adjacent to or in contact with the endothelium were counted. In all other cases, only BrdU-positive cells either within or in contact with the SM layer surrounding the vessel were counted for all lung sections. Vessel size criteria were applied in a manner identical to that used for muscularization. Computer-assisted image analysis hardware and software (Media Cybernetics, Image Pro, Silver Spring, MD) were used to
assist in the morphometric and cell proliferation measurements.

Western blot analysis and immunohistochemistry for eNOS protein. The details of Western blotting and immunohistochemistry have been published previously (18, 45). Briefly, the right lobe from each mouse lung was homogenized in homogenization buffer and centrifuged, and the protein content was analyzed by the method of Bradford (4). One hundred micrograms of protein were electrophoresed in a 7.5% SDS-polyacrylamide gel by the method of Laemmli (17) and transferred to nitrocellulose. The blot was incubated with a polyclonal anti-eNOS primary antibody (1:1,000 dilution; Transduction Laboratories, Lexington, KY) and a goat anti-rabbit horseradish peroxidase-labeled secondary antibody (1:3,000 dilution; Bio-Rad). eNOS protein signal was detected with enhanced chemiluminescence (ECL; Amersham) reagent. For immunohistochemistry, paraffin sections were processed as described in BrdU incorporation, then incubated overnight at 4°C with either a monoclonal anti-eNOS antibody (1:500 dilution; Transduction Laboratories) or a nonspecific mouse IgG (negative control). The sections were then incubated with a biotinylated anti-mouse secondary antibody (1:500 dilution; Vector Laboratories) before treatment with avidin-biotin reagent (Vector Laboratories) and visualization of the eNOS protein signal with 3'-diaminobenzidine and counterstaining with hematoxylin.

Statistical analysis. Data were analyzed for significance with multiple comparisons ANOVA. P ≤ 0.05 was considered significant.

RESULTS

Muscularization of pulmonary vessels. As depicted in Fig. 1A, WT(+/-) and eNOS(-/-) mice exposed to hypoxia exhibited significant increases in muscularization of small pulmonary vessels over normoxic control mice as early as 2 days of exposure. This increase in muscularization is reflected in the percentage of total vessels showing at least one SM cell. WT(+/-) mice exposed to hypoxia showed a roughly twofold increase in muscularized small vessels by 4–6 days of exposure compared with normoxic animals; however, the eNOS(-/-) animals exhibited a significantly lower percentage of muscularized small vessels in hypoxia compared with WT(+/-) mice at both time points. No significant differences between WT(+/-) and eNOS(-/-) mice were found with regard to percentage of fully muscularized small vessels at early time points (2, 4, or 6 days) in normoxia or hypoxia or in the degree of muscularization of medium- and large-sized vessels (data not shown).

After 3 wk of hypoxia, both WT(+/-) and eNOS(-/-) mice exhibited similar increases in the percentage of muscularized small pulmonary vessels (Fig. 1A). A trend toward an increase in muscularization of small vessels in normoxic eNOS(-/-) compared with normoxic WT(+/-) mice was detected at early time points. However, only the 3-wk cohort of eNOS(-/-) mice showed a significant increase, possibly due to the pulmonary hypertension previously observed in these mice (39). No increase in the percentage of muscularized medium and large vessels was observed in either mouse type (data not shown). WT(+/-) mice exposed to chronic hypoxia also exhibited a 1.8-fold increase in the percentage of fully muscularized small vessels (P ≤ 0.05); no corresponding increase in this parameter was detected in the hypoxic eNOS(-/-) mice (Fig. 1B). Small pulmonary vessels of hypoxic WT(+/-) animals also showed thickening of the SM wall as evidenced by the increase in %VWT compared with that in their normoxic counterparts (P ≤ 0.05; Fig. 1C). No significant increase in %VWT was found in the hypoxic eNOS(-/-) mice. Figure 2 shows examples of immunohistochemistry for SM α-actin and vWF in the pulmonary vessels of WT(+/-) and eNOS(-/-) mice exposed to normoxia or hypoxia.

Cell proliferation and BrdU labeling. As depicted in Fig. 3A, significant elevations (2.0- to 3.5-fold) in the
percentage of small pulmonary vessels containing BrdU-positive cells were detected at 4 days of hypoxic exposure in both WT(+/+) and eNOS(−/−) mice, and the percentage remained elevated at 6 days compared with that in normoxic control mice. Elevations in the percentage of small vessels with BrdU-positive cells in eNOS(−/−) mice in hypoxia were significantly less than those observed in the WT(+/+) animals at both 4 and 6 days. Additionally, the number of BrdU-positive cells per positive small vessel increased in hypoxia versus normoxia at 4 and 6 days (Fig. 3B) in both the WT(+/+) and eNOS(−/−) mice; again the increases in the eNOS(−/−) mice were less than those observed in the WT(+/+) mice. In medium-sized vessels, increases in BrdU labeling in hypoxia were detected as early as 2 days of exposure, with increases in the number of BrdU-positive cells per positive vessel significantly elevated in both mouse types. These increases persisted through the day 6 time point, with less proliferation detected in the hypoxic eNOS(−/−) mice compared with WT(+/+) mice at 6 days (Fig. 3C). Large vessels exhibited increases in the average number of BrdU-positive cells per positive vessel at 4 and 6 days of hypoxia for both WT(+/+) and eNOS(−/−) animals, with significantly less proliferation detected in the eNOS(−/−) mice at 6 days of hypoxia (Fig. 3D). No differences in proliferative indexes were detected at 3 wk of exposure between normoxia and hypoxia or between the mouse types (data not shown). Examples of immunohistochometric staining for BrdU-positive cells in the pulmonary vasculature in normoxia and hypoxia are provided in Fig. 4.

Western blot analysis and immunohistochemistry for eNOS protein. As depicted in Fig. 5, A and B, exposure of WT(+/+) mice to hypoxia resulted in a significant upregulation of eNOS protein in whole lung homogenates compared with normoxic mice at all time points examined (P < 0.05). Peak increases in eNOS protein expression were found at 6 days of hypoxic exposure, and expression remained elevated after 3 wk. No evidence of eNOS protein was detectable in lung homogenates from eNOS(−/−) mice in either normoxia or hypoxia (data not shown) as previously described (37). Immunostaining for eNOS protein in lung sections of WT(+/+) mice revealed a significant upregulation of eNOS in the small pulmonary vessels of hypoxia-exposed mice compared with normoxic mice after 3 wk of exposure (Fig. 5C). In contrast to findings in the rat (45), ~50% of small vessels in the mouse exhibited
positive eNOS staining in normoxia. Examples of eNOS immunohistochemistry are provided in Fig. 6.

DISCUSSION

The underlying cellular and molecular mechanisms of hypoxia-induced pulmonary hypertension have been the subject of intense scrutiny. Various studies (7, 13, 14, 39–41, 45, 46) have provided conflicting data regarding the potential role of NO in SM cell proliferation in the pulmonary vascular remodeling induced by hypoxia. In a rat model of chronic hypoxia, upregulation of eNOS in the small vessels of the lung precedes and progresses with the time course of muscularization of these vessels (45). The development of a mouse model of pulmonary vascular remodeling and the use of eNOS(−/−) mice to determine whether eNOS-generated NO is a mediator of vascular remodeling induced by hypoxia has been an area of intense recent interest. Our results demonstrate that lack of eNOS protein and interruption of endothelium-derived NO produce a defect in the remodeling response to chronic hypoxia in the small resistance vessels in the pulmonary vasculature. This deficiency in remodeling manifests itself early in the hypoxic time course and results in decreases in SM cell proliferation and in the percentage of small vessels showing muscularization compared with those in hypoxic WT(+/+) mice. Recent studies lent further support to the concept of a role for NO in vascular remodeling. Left common carotid artery ligation in rats produced elevations in expression of eNOS protein in the right carotid, which correlates with remodeling of this vessel (24). Strengthening this hypothesis, N-nitro-L-arginine methyl ester (L-NAME) treatment diminishes vascular remodeling in rabbits after creation of an arteriovenous fistula (43), and studies utilizing mice with targeted disruption of the eNOS locus have shown that the absence of eNOS induces elevations in pulmonary pressure (39) and abnormal remodeling of the carotid artery in response to decreased flow (34). It is also apparent from our results that other compensatory mechanisms exist in the lung vasculature to counterbalance the lack of eNOS-derived NO as indicated by our data showing that by 3 wk of hypoxic exposure, eNOS(−/−) mice
Fig. 4. Immunohistochemistry for BrdU and vWF in the lungs of WT(+/+) and eNOS(−/−) mice exposed to normoxia or hypoxia. BrdU-positive cells (thick arrowheads) are labeled blue, and endothelium (vWF; thin arrowheads) is labeled brown. A and B: representative microphotographs of lungs from WT(+/+) mice exposed to 4 days of normoxia and hypoxia, respectively. C: micrograph of lungs from an eNOS(−/−) mouse after 4 days of hypoxia. Note the proliferating vascular cells in hypoxia in small- and medium-sized vessels in B and C. Original magnification, ×200.

Fig. 5. Western blot analysis of eNOS protein from lung homogenates of WT(+/+) mice. A: protein signal for eNOS generated by chemiluminescence after normoxia and hypoxia. Nos. at left and right, molecular mass. B: results from densitometry of the protein bands. C: quantitation of the percentage of small pulmonary vessels positive for eNOS protein in WT(+/+) mice. *Significant increase, P < 0.05.
exhibited elevations in the percentage of muscularized small vessels similar to those observed for the WT(+/+) mice. In a previous study from our laboratory (32), we observed upregulation of iNOS protein expression in lung homogenates of eNOS(−/−) mice exposed to both normoxia and chronic hypoxia. This enhanced expression of an alternative isoform of NOS in eNOS(−/−) animals may compensate, in part, for the lack of NO produced by deletion of the endothelial isoform.

An interesting observation from this study is that after long-term hypoxic exposure (3 wk), deletion of eNOS seemed primarily to inhibit progression from a partly muscularized pulmonary vessel to a fully muscularized one and also prevented thickening of the SM wall in fully muscularized small vessels. Our results are in contrast to recent studies (7, 40) of hypoxia-induced pulmonary hypertension that detected increases in vascular remodeling in eNOS(−/−) mice compared with WT(+/+) mice. However, both of these studies evaluated the time response of remodeling (6 wk of hypoxia exposure) and utilized eNOS(−/−) mice of a different genetic background (C57BL/6-SV129) than those used in our experiments. They also examined either a single WT control strain (40) or nonbackcrossed hybrid WT animals (7). Studies have demonstrated that controlling for genetic background is essential in vascular research, including gene deletion. It has been very well documented that there are strain differences in resting levels of arterial blood pressure (36), in susceptibility to atherosclerosis (31, 38), and in angiogenesis (42). The eNOS(−/−) mice used in our study were backcrossed extensively (seven generations) to the parental C57BL/6 strain to minimize potential variability introduced by the use of mice of mixed genetic background. Our control mice more extensively remodeled the pulmonary vasculature in hypoxia than the control mice used by Steudel et al. (40) and Fagan et al. (7), which accounts, in part, for the discrepancies in results. Additionally, the study by Fagan et al. used a hypobaric hypoxic exposure protocol, which may have had an impact on their results. It is also possible that different strains of mice and, more importantly, the effect of mixing genetically different strains may produce varying levels of growth factors, receptors, or other physiologically important molecules that could alter the extent of remodeling in response to hypoxia. Clearly, this is an area that deserves further study.

Our study is novel and distinct from previous studies in the examination of the role of cell proliferation in pulmonary vascular remodeling in all sizes of vessels.

Fig. 6. Immunohistochemistry of eNOS protein in WT(+/+) mouse lungs exposed to normoxia or hypoxia for 3 wk. Arrows, eNOS staining in pulmonary vessels in mice exposed to normoxia (A) or hypoxia (B). C: microphotograph of a lung section from a WT(+/+) mouse exposed to hypoxia and used as a negative control. Original magnification, ×200.
and of how eNOS deletion affects proliferative indexes in vivo. Analysis of data from the early time points indicates the importance of vascular cell proliferation in remodeling of the mouse pulmonary vasculature in response to more acute hypoxic exposures. Our findings suggest that the levels of NO generated by eNOS in vivo under hypoxic conditions may be proproliferative for vascular SM cells or precursors. This hypothesis is reinforced by the pronounced elevations in indexes of cell proliferation observed in the small pulmonary vessels at 4 and 6 days of exposure and the diminished proliferation in the vasculature of eNOS(−/−) mice in hypoxia at these early time points compared with those in WT(+/+) mice. The decreased vascular cell proliferation correlates with less muscularization of small vessels in this mouse type than that observed in the WT(+/+) animals and provides a mechanistic explanation for the decreased remodeling. These results differ from previous studies (25, 34) that examined vascular cell proliferation in eNOS(−/−) mice. However, these studies focused on large systemic arteries and utilized carotid artery ligation (34) and femoral cuff (25) models, both of which are significantly different from the methodology used in our study, making direct comparison of results problematic. A recent study by Ulibarri and coworkers (44) showed that NO concentration may influence its pro-versus antiproliferative response. They found that low levels (5–10 μM) of sodium nitroprusside and S-nitroso-N-acetylenicollamine stimulated myoblast proliferation and that a high level (50 μM) of S-nitroso-N-acetylenicollamine inhibited proliferation. The exact mechanism for the role of NO induction of cell proliferation is currently unknown. Reports from other studies have implicated NO as a mediator of cell proliferation during angiogenesis. There is reduced angiogenesis in response to tissue ischemia (27), impaired wound healing, and angiogenesis (19) in eNOS(−/−) mice, implying that NO may act through vascular endothelial growth factor (10, 30) and activation of mitogen-activated protein kinase (16).

Although the role of NO in modulating proliferation of vascular cells is controversial (6, 23, 35, 46), most studies showing that NO is antiproliferative in vascular SM cells in vitro made use of cells at high passage (22), in which cGMP-dependent protein kinase, a major downstream effector of NO signaling, is downregulated. Decreased cGMP-dependent protein kinase expression correlates with a dedifferentiated phenotype, and stimulation of these cells with NO results in cGMP-mediated activation of the cAMP-dependent protein kinase pathway and inhibition of mitogenesis (3, 5). Furthermore, exposure of primary cultures of aortic SM cells to NO donors enhances the mitogenicity of basic fibroblast growth factor (14), implying that NO is a proproliferative molecule in fully differentiated vascular cells.

An intriguing result of our study is that muscularization of small lung vessels begins as early as 2 days of exposure to hypoxia, with no accompanying increase in vascular cell proliferation. This finding indicates the possibility of two distinct processes initiated sequentially in the pulmonary vasculature as it adapts to hypoxic conditions. The early acute phase may involve differentiation and/or migration (15) of SM cell precursors such as pericytes or fibroblasts, which provide the initial muscularization of previously nonmuscular small vessels. The later phase, which begins between 2 and 4 days of hypoxia, involves induction of vascular cell proliferation to further muscularize these resistance vessels. Also of note is the finding that elevations in proliferating SM cells were detected in both medium and large vessels at 4 and 6 days of hypoxia compared with those in normoxic control mice. However, these classes of vessels showed no corresponding increases in muscularization, likely due to counteracting mechanisms such as apoptosis, which prevents increases in muscularity of SM walls. This topic is presently under investigation in our laboratory. Our results suggest that endogenous eNOS activity is critical for the proliferation of vascular SM cells in hypoxia-induced pulmonary hypertension. It is possible, however, that the amount of NO delivered, local levels of NO achieved, and/or temporal requirements may be different from endogenous synthesis when NO donors are used to compensate for a deficiency of authentic NO.

In conclusion, our studies indicate that NO derived from eNOS is a mediator of vascular cell proliferation and muscularization of small pulmonary vessels in response to hypoxia and support previous observations from our laboratory and others (18, 34, 45) regarding the role of eNOS in vascular remodeling. Lack of eNOS leads to decreased hyperplastic potential and muscularization of small vessels during more acute exposures. Furthermore, NO deficiency inhibits progression from partly to fully muscularized vessels and abrogates the thickening of SM walls seen in WT(+/+) mice exposed to prolonged hypoxic conditions. However, pulmonary vessels in eNOS(−/−) mice do undergo remodeling, including proliferation and muscularization, in response to hypoxia, indicating that alternative mechanisms such as iNOS upregulation exist in these mice, enabling them to adapt to the lack of NO and to conditions of low oxygen tension.

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