Inhibition of compensatory lung growth in endothelial nitric oxide synthase-deficient mice

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Leuwerke, Shari M., Aditya K. Kaza, Curtis G. Tribble, Irving L. Kron, and Victor E. Laubach. Inhibition of compensatory lung growth in endothelial nitric oxide synthase-deficient mice. Am J Physiol Lung Cell Mol Physiol 282: L1272–L1278, 2002.—Pneumonectomy results in rapid compensatory growth of the remaining lung and also leads to increased flow and shear stress, which are known to stimulate endothelial nitric oxide synthase (eNOS). Nitric oxide is an essential mediator of vascular endothelial growth factor-induced angiogenesis, which should necessarily occur during compensatory lung growth. Thus our hypothesis is that eNOS is critical for compensatory lung growth. To test this, left pneumonectomy was performed in eNOS-deficient mice (eNOS−/−) and compensatory growth of the right lung was characterized throughout 14 days postpneumonectomy and compared with wild-type pneumonectomy and sham controls. Compensatory lung growth was severely impaired in eNOS−/− mice, as demonstrated by significant reductions in lung weight index, lung volume index, and volume of respiratory region. Also, pneumonectomy-induced increases in alveolar surface density and cell proliferation were prevented in eNOS−/− mice, indicating that eNOS plays a role in alveolar hyperplasia. Compensatory lung growth was also impaired in wild-type mice treated with the nitric oxide synthase inhibitor Nω-nitro-L-arginine methyl ester. Together, these results indicate that eNOS is critical for compensatory lung growth.

IN 1892, IT WAS FIRST DOCUMENTED that after pneumonectomy (removal of a lung) in experimental animals, the remaining lung increases in size (9). This adaptive growth, referred to as compensatory lung growth, results in restoration of lung function and total lung volume, compliance, mass, DNA, protein, alveolar number, and essentially normal lung cell populations (2, 3, 15). Various pathways play a role in this growth response, including changes in lung inflation, blood flow, and release of growth-promoting substances such as growth factors. There is evidence that type II epithelial cells play a role because type II cells isolated from the lungs of pneumonectomized rats exhibit metabolic changes typical of accelerated cell growth (16). Brody (1) reported from studies in rats and mice that the process of compensatory lung growth is completed 14 days postpneumonectomy when there is an early phase of cell proliferation within the first several days after surgery, followed by an increase in functional capacities. It was also documented by Brody (1) and Cagle and Thurlbeck (3) that the protein-to-DNA ratio was essentially constant over the postoperative time, indicating that the lung mass had been restored by hyperplastic growth (increase in cell number) rather than hypertrophic growth (increase in cell size). Despite these and other studies, the molecular mechanisms that trigger and regulate compensatory lung growth remain unknown.

Nitric oxide (NO) is synthesized from L-arginine by three nitric oxide synthase (NOS) isoenzymes. Endothelial nitric oxide synthase (eNOS) and neuronal NOS are constitutively expressed in endothelial cells and neurons, respectively, and generate small amounts of NO on activation by Ca2+, whereas inducible nitric oxide synthase (iNOS) is induced by proinflammatory cytokines in various cell types, resulting in a sustained high output of NO. NO has been shown to stimulate the proliferation and migration of endothelial cells (11). It is now apparent that vascular endothelial growth factor (VEGF) effects are selectively linked to the eNOS pathway. VEGF is a potent angiogenic growth factor that induces endothelial cell proliferation, migration, and tube formation and is released by a variety of cell types, including lung epithelial cells (14). Morbidelli et al. (11) demonstrated that VEGF stimulates proliferation of endothelial cells through the production of NO. Ziche et al. (20) concluded that NO is a downstream imperative of VEGF-induced angiogenesis and proposed that the eNOS/guanylate cyclase pathway is a potential target for controlling tumor angiogenesis.

The long-term objective of our research is to understand the molecular mechanisms that trigger and regulate compensatory lung growth, focusing currently on NO and its role in VEGF-induced angiogenesis and lung epithelial cell proliferation. The growth of new tissue during lung regeneration likely requires angiogenesis; pulmonary regeneration; pneumocytes; alveolar proliferation.

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genesis to nourish the newly formed tissue as well as epithelial cell proliferation to support alveolar hyperplasia. After pneumonectomy, it is also likely that increased vessel shear stress within the remaining tissue and the initiation of growth factor-regulated angiogenesis will promote and regulate compensatory lung growth, at least partially, through the eNOS signaling pathway. From studies done in the liver, we know that after partial hepatectomy, increased blood flow and shear stress stimulate eNOS expression, and enhanced cytokine production triggers iNOS upregulation (5).

In this study, we focus on the role of eNOS in compensatory lung growth by hypothesizing that eNOS-generated NO is a critical mediator of compensatory lung growth. To test our hypothesis, knockout mice deficient in eNOS (eNOS−/−) were used to study NO-mediated mechanisms of postpneumonectomy lung growth.

**METHODS**

**Animals.** All animals were mature, age-matched (14–16 wk) male and female mice weighing ~25 g each. Wild-type C57BL6 mice were purchased from Hilltop. The eNOS−/− mice were generated by Shesely et al. (18) and were backcrossed onto the C57BL6 genetic background at least seven generations to be directly comparable to the C57BL6 wild-type mice. The eNOS−/− mice did not differ in weight from similar aged wild-type mice. Animal acquisition was under the supervision of the Department of Comparative Medicine and a licensed veterinarian. All animals received humane care in compliance with the Principles of Laboratory Animal Care formulated by the National Society for Medical Research and the Guide for the Care and Use of Laboratory Animals prepared by the National Academy of Science and published by the National Institutes of Health (NIH Publication No. 85-23, revised 1985).

**Animal surgery.** Mice were divided into three groups: wild-type sham controls (WT-S), wild-type pneumonectomy (WT-P), and eNOS pneumonectomy (eNOS-P). Each group was then subdivided into five subgroups on the basis of the postoperative survival times: immediate (time 0); 1 day, 3 days, 7 days, and 14 days (n = 10/group). All animals were anesthetized with ketamine and xylazine. Injections were made intraperitoneally along with an injection of 0.5 ml of saline to alleviate any blood volume loss. Each mouse was intubated with a 24-gauge catheter. The surgical apparatus consisted of a pressure-regulated rodent ventilator (Kent Scientific) during the surgery (~10 min) and were then moved to recover on room air using an identical ventilator until spontaneous respiration began. The animals were allowed to feed and drink freely and were maintained in a controlled environment. After the assigned postoperative survival time, the animals were again anesthetized, and a final body weight of each animal was recorded. The right lungs of half of the animals were removed, patted dry, weighed, frozen in liquid nitrogen, and then stored at −80°C for molecular analysis.

The remaining animals in each group received intratracheal fixation with 10% buffered Formalin. Here, the trachea was cannulated, and the trachea and lung with surrounding tissue were excised, patted dry, and weighed. The lung was then inflated with 10% buffered Formalin at a constant pressure of 25 cmH2O. The lung was then tied off from the cannula, submersed in 10% buffered Formalin, and allowed to fix for ~60 min. The excess tissue was then trimmed off, weighed, and this weight was subtracted from the initial lung weight to obtain a final lung weight. Lung volumes were determined using the volume displacement technique as described by Scherle (17). The lungs were then cut medially in half, placed in 70% ethanol, and stored at 4°C overnight. The lungs were then embedded in paraffin, and 5-μm sections were stained with hematoxylin and eosin for morphometric analysis. The lung weights and volumes were expressed as a ratio to the final body weight [lung weight index (LWI)] and lung volume index (LVI), respectively, to correct for variability in animal size.

**Lung morphometry.** Lung morphometry was performed using a three-level sampling technique described by Davies (4) and Wandel et al. (19), a point counting technique described by Gil (7), and as previously described by our laboratory (9). A 42-point test reticule attached to a Nikon Eclipse E400 microscope was used. This technique involved three levels of study. Level one was performed at ×50 magnification. The number of lattice points that fell on intra-acinar airspace and the intervening tissue was counted and designated as Pp. Nonrespiratory regions, which included extra-acinar airways and vessels >0.5 mm in diameter were ignored. The volume of the respiratory region (Vr) was calculated using the equation Vr = Pr/42 × 100, which represents the percent of lung comprised of respiratory (alveolar) tissue.

The second level of analysis, which was performed under ×200 magnification, counted the number of lattice points that fell in respiratory airspaces and was designated as Pr. The volume of the respiratory airspace (Vr) was then calculated using the equation Vr = Pr/42 × 100. The volume of respiratory tissue (Vt) was determined by counting the number of lattice points that fell in respiratory tissue (Prt) using the equation Vt = Pr/42 × 100. These values represent the percent of the lung that is comprised of respiratory alveolar airspace and tissue, respectively.

The last level of analysis, which was also performed under ×200 magnification, counted the number of test lines that intercepted the airspace–epithelial interfaces and was designated as L. Alveolar surface density (Sv), which expresses the alveolar surface area per unit volume (cm2/cm3), was determined using the equation $Sv = 2d \times L/Vt$, where $d$ = length of the test grid line (85 μm) and $Pp$ = total number of test points on the lung parenchyma, which is 42.

The total volume of the respiratory region (TVr) was calculated as $TVr = Vr/ \times Vt$, where $Vt$ = total lung volume. $TVr$ is a measure of the total volume of the alveoli and intervening gas exchange tissue. The total volume of the respiratory airspace (TVa) was calculated as $TVa = Vra/ \times Vt$. This is a measure of alveolar airspace volume in the lung. Total volume of respiratory tissue (TVt) was calculated as $TVt = Vt/ \times Vt$. This is a measure of alveolar tissue volume in the lung.

**Cell proliferation index.** Bromodeoxyuridine (BrDU; 80 mg/kg) was injected intraperitoneally 15 h before lung har-
vest. A BrDU detection kit (BD Pharmingen kit) using biotinylated anti-BrDU monoclonal antibody (1:10) was used as instructed for immunohistochemistry of parafin-embedded lung sections. Slides were then studied using light microscopy. Cell proliferation index (CPI) was determined by counting the number of stained nuclei per 1,000 total cells and multiplying by 100. Cells adjacent to large airways and vessels were ignored to calculate the CPI of alveolar tissue only. This procedure determines the percentage of alveolar cells that are undergoing cell division.

**eNOS protein expression.** Western blot analysis was performed to determine eNOS protein expression. Total lung protein (100 μg) was fractionated on a 6.5% sodium dodecyl sulfate polyacrylamide gel and then transferred to nitrocellulose using an electrophoretic transfer cell (Bio-Rad). The blot was then blocked with 5% milk and incubated with primary rabbit anti-mouse eNOS antibody (1:2,500, Transduction Laboratories) for 2 h at room temperature and washed with 50 mM Tris-HCl, pH 7.4, 150 mM NaCl, and 0.1% Tween. The blot was then incubated for 1 h with secondary anti-rabbit antibody that was coupled to horseradish peroxidase. Protein bands were visualized by chemiluminescence (ECL, Amersham) and quantitated by computerized densitometry (Alpha Innotech).

**NOS inhibitor.** Wild-type mice (n = 14) underwent left pneumonectomy, as described above, and were allowed to survive for 14 days. Beginning on the day of surgery, half of the mice were injected intraperitoneally twice daily with Nω-nitro-L-arginine methyl ester (L-NAME; Sigma, 40 mg/kg), a potent, nonspecific inhibitor of NOS. The other half were injected with Nω-nitro-D-arginine (D-NAME; Sigma, 40 mg/kg), an inactive control. At 14 days postpneumonectomy, the right lung of each mouse was harvested and Formalin fixed as described above. Lung weight, volume, and morphometry measurements were calculated as described above. Serum was also collected at the time of harvest, and nitrate plus nitrite levels were measured using a nitrate/nitrite colorimetric assay kit (Cayman Chemical) as instructed.

**Statistical analysis.** Measurements are reported as means ± SE of the mean. Two-way analysis of variance was used to determine whether a difference exists between study groups. A P value of 0.05 or less was used to indicate significant differences. A Bonferroni multiple-comparison test was used when appropriate.

**RESULTS**

**eNOS protein expression.** To determine whether eNOS is upregulated during compensatory lung growth, eNOS protein expression was measured in whole lung tissue homogenate by Western blot at 0, 1, 3, 7, and 14 days postpneumonectomy in WT-P mice. eNOS expression was significantly increased at days 3, 7, and 14 postpneumonectomy, peaking at day 7 (Fig. 1). eNOS protein expression remained relatively constant in WT-S mice (Fig. 1), and eNOS protein was undetectable in eNOS-P mice by Western blot analysis, as would be expected (not shown).

**LWI.** As expected, LWI increased throughout the 14-day time period in WT-P mice (Fig. 2). At 14 days postpneumonectomy, LWI was significantly increased in WT-P mice (5.83 ± 0.25 mg/g) compared with WT-S controls (3.50 ± 0.11 mg/g). Of particular interest, eNOS-P mice had significantly reduced LWI (3.90 ± 0.23 mg/g) compared with WT-P and WT-S controls (5.83 ± 0.25 mg/g).
0.22 mg/g) compared with WT-P mice at day 14 and were similar to the WT-S mice. LWI of sham-operated animals remained relatively constant. Ratios of lung wet-to-dry weights were similar at 14 days postpneumonectomy in WT-P and WT-S, indicating that the increase in lung weight was not due to water content (data not shown).

LVI. As expected, LVI of WT-P mice was significantly increased at 3, 7, and 14 days postpneumonectomy with maximal LVI at 14 days (29.5 ± 2.2 ml/kg) compared with WT-S controls (9.2 ± 1.2 ml/kg; Fig. 2). Interestingly, eNOS-P mice displayed a significant impairment of LVI at 3, 7, and 14 days postpneumonectomy compared with WT-P mice (Fig. 2). At 14 days postpneumonectomy, LVI was 14.2 ± 1.4 ml/kg in eNOS-P mice, which was not significantly different from eNOS-P at day 0 and WT-S at day 14.

CPI. Alveolar CPI was determined by BrDU immunohistochemistry as described in METHODS and as illustrated in Fig. 3 (bottom). Basal alveolar CPI was 3.6 ± 0.3% in WT-P mice (day 0), which was significantly elevated at 3, 7, and 14 days postpneumonectomy (Fig. 3). Peak values were achieved at 7 days postpneumonectomy when CPI in WT-P mice reached 17.1 ± 1.4% vs. 3.7 ± 1.0% in WT-S. Most notably, alveolar CPI in eNOS-P mice was similar to WT-S controls and did not increase significantly at any time point (Fig. 3).

\( V_{vr} \) \( V_{vr} \) is the percent of the lung that is active in gas exchange (i.e., alveolar regions). There was a significant increase in \( V_{vr} \) in WT-P mice at days 3, 7, and 14 compared with WT-S controls (Fig. 4). At 14 days postpneumonectomy, \( V_{vr} \) in WT-P mice reached 93.2 ± 1.8% compared with WT-S controls (71.6 ± 2.3%). \( V_{vr} \) was significantly impaired in eNOS-P mice at day 14 (83.8 ± 1.3%) compared with WT-P (Fig. 4). Note that although \( V_{vr} \) in eNOS-P mice was higher than WT-S at 14 days, it was similar to the day 0 time point.

\( TV_{vr} \). \( TV_{vr} \) is the total volume of the lung that is comprised of respiratory region (i.e., alveoli). WT-P mice displayed a significant increase in \( TV_{vr} \), at days 1, 3, 7, and 14 (Fig. 4). At 14 days postpneumonectomy, \( TV_{vr} \) in WT-P mice reached 619.6 ± 30.7 \( \mu \)l compared with WT-S (176.1 ± 14.3 \( \mu \)l). Most notably, \( TV_{vr} \) of eNOS-P (332.0 ± 30.0 \( \mu \)l) was significantly lower than WT-P mice at 14 days postpneumonectomy. Although

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**Fig. 3.** Alveolar cell proliferation index (CPI) at various days after surgery. The percent of alveolar cells proliferating were evaluated by immunohistochemical analysis of bromodeoxyuridine (BrDU) nuclear incorporation. *Top:* comparison of alveolar CPI in the various groups at the indicated times postsurgery. *P* < 0.001 WT-P vs. all. *Bottom:* representative lung sections illustrating BrDU immunohistochemical staining (×400 magnification, hematoxylin counterstain). A: WT-S at day 7. B: WT-P at day 7. C: eNOS-P at day 0. D: eNOS-P at day 7. Note the increased alveolar CPI (brown-stained nuclei) visible in WT-P (B).

**Fig. 4.** Volume of respiratory region \( (V_{vr}, A) \) and total volume of respiratory region \( (TV_{vr}, B) \) in mouse lungs at various days after surgery. *P* < 0.001 WT-P vs. all. *P* < 0.001 eNOS-P vs. WT-S.
TV\textsubscript{vr} in eNOS-P mice at day 14 was higher than in WT-S, it was similar to TV\textsubscript{vr} in eNOS-P mice at day 0 (300.7 ± 5.7 \mu l). The volume of respiratory region in WT-S controls remained stable.

TV\textsubscript{ra} and TV\textsubscript{rt}. TV\textsubscript{vr} consists of TV\textsubscript{ra} and TV\textsubscript{rt}. Similar to the pattern observed with TV\textsubscript{vr}, TV\textsubscript{ra} and TV\textsubscript{rt} were significantly increased in WT-P mice, particularly at 7 and 14 days, compared with WT-S controls (Fig. 5). Most notably, eNOS-P mice (224 ± 22.5 \mu l) had significantly lower TV\textsubscript{ra} than WT-P mice (442 ± 23.7 \mu l) at day 14 (Fig. 5). A similar pattern was seen in TV\textsubscript{rt} at day 14 when TV\textsubscript{vr} in eNOS-P (87.0 ± 11.7 \mu l) was significantly impaired compared with WT-P (153 ± 4.5 \mu l; Fig. 5). Together, these results indicate that eNOS-P mice have severely impaired regeneration of alveolar and tissue volumes.

S\textsubscript{v}. S\textsubscript{v} is an expression of the alveolar surface area per unit volume (cm\textsuperscript{2}/cm\textsuperscript{3}). S\textsubscript{v} in WT-P mice was significantly increased at 3, 7, and 14 days postpneumonectomy, peaking at 3 days (Fig. 6). At 3 days postpneumonectomy, S\textsubscript{v} in WT-P mice reached 518 ± 11.8 cm\textsuperscript{-1} compared with WT-S controls (469 ± 8.3 cm\textsuperscript{-1}). Interestingly, eNOS-P mice had significantly lower S\textsubscript{v} than WT-P mice at 3 days postpneumonectomy (441 ± 17.0 cm\textsuperscript{-1}) and never achieved levels above WT-S controls at any time point (Fig. 6). The initial increase in S\textsubscript{v} observed in WT-P mice suggests alveolar hyperplasia followed thereafter by a gradual period of hyper trophy.

NOS inhibition. To further support our hypothesis that NO is critical for postpneumonectomy lung growth, wild-type mice were treated with a NOS inhibitor (L-NAME) during the 14-day postpneumonectomy period. A second group of wild-type mice were treated with \textit{d}-NAME as a control group. Serum nitrate plus nitrite levels were significantly reduced in L-NAME-treated mice (2.81 ± 0.46 \mu M) vs. \textit{d}-NAME-treated mice (5.46 ± 0.56 \mu M) at day 14 (P < 0.01). Here, it was observed that mice treated with \textit{l}-NAME had significantly impaired lung growth, as illustrated by reductions in LWI and LVI compared with \textit{d}-NAME control group (Fig. 7). In addition, morphometric evaluation indicated that inhibition of NOS by \textit{l}-NAME significantly reduced V\textsubscript{vr}, TV\textsubscript{vr}, and S\textsubscript{v} (Fig. 8). These results mirror closely those obtained with the eNOS-P mice and indicate that compensatory mechanisms in NO signaling, as a result of inherent gene deficiency, are probably not involved in the impaired compensatory lung growth observed in the eNOS\textsubscript{−/−} mice.

DISCUSSION

The treatment of lung disease often requires pneumonectomy or lobectomy, usually resulting in compensatory growth of the remaining lung in infants and young children. Therefore, understanding the controlling factors of compensatory lung growth could allow us to minimize the stresses experienced by the remaining lung and shorten the time required for new tissue growth. Also, understanding compensatory lung growth is critical when considering that the shortage of donor lungs is a major obstacle hindering the widespread application of lung transplantation surgery.
Being able to manipulate lung growth will be vital for bringing about new and better treatments for lung disease and lung injury.

Previous studies in eNOS−/− mice have shown that effective wound repair requires growth factor-stimulated angiogenesis accompanied by adequate rates of NO production. Lee et al. (10) showed that eNOS−/− mice had significantly delayed wound closure compared with WT controls, and this group also confirmed in vitro that eNOS is required for proper endothelial cell migration, proliferation, and differentiation, events necessary for new vessel growth. This indicates that NO generated by eNOS is necessary for proper growth factor-stimulated angiogenesis both in vitro and in vivo. Similarly, we anticipate that compensatory lung growth requires angiogenesis.

In the present study, we have demonstrated for the first time that NO is a necessary mediator of compensatory lung growth. We studied the role of NO in compensatory lung growth at various time points ranging from 0 to 14 days postpneumonectomy utilizing eNOS−/− mice. In the lung, eNOS has been detected in both airway epithelial cells and endothelial cells where it is partly responsible for providing basal pulmonary vascular tone (6). We first sought to measure changes in eNOS protein expression in response to pneumonectomy. We anticipated an increase in eNOS expression due to increased blood flow and shear stress to the remaining tissue. We found that eNOS protein expression was significantly increased in wild-type mice at 3, 7, and 14 days postpneumonectomy, indicating the upregulation of eNOS during compensatory lung growth.

Ofulue et al. (12) studied the role of calmodulin, a ubiquitous protein and stimulator of eNOS activity, in compensatory lung growth after pneumonectomy. They found that lung calmodulin content and activity were significantly increased at 1 and 2 days postpneumonectomy in rats. This increase in calmodulin activity precedes the initial increase in lung mass and volume observed in multiple studies, which is typically ~3–7 days postpneumonectomy. Treatment of the pneumonectomized rats with a calmodulin antagonist, which would inactive eNOS, resulted in diminished calmodulin activity along with a reduction in lung mass. These results support our findings that eNOS−/− mice have impaired lung growth. We found that both LWI and LVI in eNOS−/− mice failed to achieve similar increases that were observed in wild-type mice throughout the 14-day period. Wet-to-dry weight ratios were similar in WT-P and WT-S groups, indicating that the increase in lung mass after pneumonectomy was not due to an increase in water weight.

To determine whether alveolar hyperplasia was impaired in eNOS−/− mice, alveolar CPI was determined. We found that the CPI was significantly increased in wild-type mice where peak CPI occurred at 7 days postpneumonectomy, similar to results documented by Brody (1) and Cagle and Thurlbeck (3), who noted peak CPI at 5–7 days postpneumonectomy. Surprisingly, we found no increase in CPI above sham controls after pneumonectomy in eNOS−/− mice, indicating impairment in compensatory growth via lack of alveolar cell proliferation.

The percent of the lung comprised of gas exchange units (V vr, i.e., alveolar regions) was greatly reduced in eNOS−/− animals compared with wild-type mice after pneumonectomy. This was most clearly seen by TV vr, which takes into consideration both the percent of the lung that is respiratory and the total lung volume. Here, eNOS−/− mice show severely impaired TV vr compared with wild-type mice. Because TV vr consists of both respiratory airspace and tissue, TV ra and TV rt show a similar impaired growth pattern in eNOS−/− mice. Together, compared with wild-type mice, eNOS−/− mice have significantly decreased amounts of alveolar tissue and airspace after pneumonectomy, indicative of impaired compensatory growth.

Wild-type mice undergoing pneumonectomy displayed an initial increase in S v. S v peaked at day 3 of postpneumonectomy and then decreased gradually thereafter. The early rise in S v suggests alveolar hyperplasia, followed by a subsequent gradual hypertrophic response. This was not seen in eNOS−/− mice where S v remained below sham levels. Because eNOS−/− mice had significantly lower TV ra and TV rt, it is not surprising that S v remained relatively constant, indicative of a lack in alveolar growth response.

As with all studies utilizing knockout animals, it is important to ensure that the results are actually due to the lack of NO and not to possible compensatory effects of the gene knockout. It is known that eNOS−/− mice have mild pulmonary hypertension, with pulmonary vascular resistance more than double that of wild-type mice (8). To document that the impaired lung growth in the knockout animals was not simply due to artifactual
or compensatory responses in the mice from the inherent gene deficiency, we administered a NOS inhibitor (L-NAME) to wild-type mice after pneumonectomy. We found that mice treated with L-NAME displayed a similar impairment in lung growth (illustrated by decreased LWI, LVI, and morphometric measurements) as did the eNOS+/− mice, indicating that impairment in compensatory lung growth can be achieved in wild-type mice by inhibition of NOS activity. Although we cannot rule out possible involvement of other NOS isoforms in compensatory lung growth, the fact that L-NAME did not result in more severe impairment of lung growth than in eNOS+/− mice suggests that eNOS is a major isoform of importance.

Angiogenesis of new vessels to nourish the newly formed tissue should accompany compensatory lung growth. Serum VEGF levels are significantly elevated at 3 and 7 days postpneumonectomy in wild-type mice (data not shown). Because VEGF is a potent angiogenic factor and is abundantly expressed in lung epithelium and because eNOS is known to be upregulated by VEGF stimulation, it is possible that impaired lung regeneration in eNOS+/− mice could involve a defect in the VEGF-induced angiogenesis pathway. Parenti et al. (13) have shown that the mitogenic activity of VEGF requires the activation of the mitogen-activated protein kinase (MAPK) cascade, which is mediated by NO production when VEGF receptors are activated, and that endothelial cell proliferation promoted by NO is selectively blocked by MAPK inhibition. Thus the lack of NO in eNOS+/− mice may be blunting VEGF signaling, thus impairing angiogenesis and subsequent lung growth. This could also hold true for basic fibroblast growth factor and epidermal growth factor signaling as well. Further studies are needed to test this. The angiogenic pathway is regulated, in part, by oxygen tension. It is thus possible that the acute, transient hyperoxic exposure of the mice during surgery could affect subsequent angiogenesis and growth. This seems unlikely, however, because the exposure is very short (10 min on average) and because no affects were seen in the sham-operated mice that underwent the same hyperoxic exposures during surgery.

Previous studies have described a vital role of eNOS in growth factor-induced angiogenesis. Because angiogenesis is necessary for the viability of new tissue and because lung epithelial cell proliferation is necessary for alveolar hyperplasia, we believe that eNOS may be involved in the initiation and/or regulation of compensatory lung growth, possibly through regulation of endothelial and epithelial cell growth. Based on the impairment of lung regeneration in the eNOS+/− mice and in mice-administered NOS inhibitor, our results indicate that NO is an essential mediator of compensatory lung growth.

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