Nitric oxide (NO) is produced by several nitric oxide synthases (NOS) through sequential oxidation of a terminal nitrogen of L-arginine. At least three NOS isoforms are recognized: NOS I (initially isolated in association with neuronal and epithelial tissues), NOS II (inducible and associated with macrophages), and NOS III (found extensively in endothelium and the most common lung-associated NOS). NOS I and NOS III are constitutively expressed and calcium responsive and usually produce nanomolar tissue concentrations of NO. NOS II is inducible, calcium independent, and capable of producing damaging micromolar tissue concentrations of NO. NOS may produce superoxide (O$_2^-$) as a byproduct of its enzymatic action (20).

Like molecular oxygen, NO has an unpaired outer orbital electron, is paramagnetic, and will undergo one electron reduction and oxidation. NO is known to react directly with transition metals and will interact with sulfur and iron-containing proteins as has been shown for soluble guanylate cyclase. Many other protein-RNS interactions probably occur. For example, NO may reversibly regulate cytochrome $aa_3$ (cytochrome oxidase) and other sulfur and iron-containing proteins (3, 4, 9); NO likely forms S-nitrosyl compounds with hemoglobin, and this reversible reaction could function to release NO under certain conditions (10); oxidation of hemoglobin to form met-hemoglobin and nitrate has been invoked as a common metabolic pathway for NO degradation; NO may bind to and alter the enzymatic activity of iron-sulfur enzyme proteins such as aconitase (1). The regulation of a key apoptosis factor, caspase 3, through reversible nitrosylation (15) provides a mechanism to explain an influence of NO upon apoptosis. Consistent with its influence on caspase 3, NO often is an antiapoptotic signal (23), but NO also may promote apoptosis of some cell types (17).

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The half-life of NO is a linear function of the concentration of O\textsubscript{2} and is an exponential function of its own concentration. Normal tissue concentrations of NO are thought to range from the low nanomolar range up to about 1-μM concentration under physiological conditions. Inflammatory conditions likely result in significant upregulation of NOS II with resultant high production of NO and tissue levels that may be toxic. NO is a relatively long-lived species and, therefore, able to diffuse significant distances. It is hydrophobic and thus is concentrated within lipid-rich cellular membranes (27). NO is usually considered to be acting in a paracrine function such as occurs in the well-described situation where endothelial cell-derived NO produced by NOS III acts to increase cyclic guanine monophosphate (cGMP) in adjacent vascular smooth muscle cells, thus causing smooth muscle relaxation and vasodilation (11). Additional pathways that invoke transduction of NO through S-nitrosyl compounds for targeted NO delivery to areas of hypoxia are also possible (10).

Branching morphogenesis of the mammalian lung is an early developmental event beginning about postnatal day 10 in the rat and week 3 in human. A process of specified branching defines the formation of the major bronchi. The remainder of the conducting airways are formed according to a dichotomous branching pattern in the human and a monopodial pattern in the rat. All of the conducting airways are completed by about midgestation. Cellular signals controlling the branching pattern are not well known, but it is clear from genetic experiments in mice that the fibroblast growth factor (FGF) family and their receptors are critical to the branching process (18). General patterns of cellular proliferation and modification of structures through a process of apoptosis certainly occur during lung development, although many details remain to be discovered. We postulate that NO contributes to lung development by multiple mechanisms that might include promotion of cell growth or effects on apoptosis as well as modulation of expression of key signaling molecules including members of the FGF superfamily.

The expression of NOS in multiple tissues in a time- and space-dependent manner suggests that NO may be important in directing the development of these tissues. Mice with a targeted deletion of NOS III have a lung phenotype that is expressed during postnatal development. Such animals are hypersensitive to mild hypoxia and develop pulmonary hypertension as well as an abnormal muscularization of small pulmonary arteries when they, homozygous or heterozygous, are raised in hypoxic conditions (6). These physiological and anatomical consequences confirm at least one role for NOS III in regulating pulmonary vascular resistance and its response to hypoxia. The NOS III effect to enhance smooth muscle expression in small pulmonary vessels is consistent with a role for NO in the regulation of vascular smooth muscle development. Another model of abnormal lung growth has been described in the fawn-hooded rat, an inbred rat strain bearing a partial deficiency of NOS III activity. These animals are hyperresponsive to mild hypoxia, and they demonstrate an abnormality in postnatal alveolarization (13, 14).

We began these studies to test whether an altered concentration of NO might affect a bioassay of branching morphogenesis carried out with explanted fetal rat lung in organ culture. We hypothesized that elevated concentrations of NO would inhibit branching due to the production of toxic reactive species. We expected that any effect of NO would be dose dependent. High doses, especially, were expected to be toxic. Herein we report a dose-dependent stimulation of lung branching by exogenous NO, an apparent toxicity at high doses and inhibition of branching by pharmacological inhibition of NOS activity. These effects were not mediated by cGMP.

**METHODS**

**Animals.** Timed-pregnant Sprague-Dawley rats were obtained from Zivic Labs (Pittsburgh, PA). Day of birth for these animals is gestational day (gd) 22. The dams were housed in individual cages and given sufficient dust-free bedding to allow for normal nesting behavior. For collection of fetal lungs, dams were deeply anesthetized with 50 mg/kg ip pentobarbital sodium. Fetuses were removed from their amniotic sacs and placed on a moistened petri dish in contact with melting ice. Lungs were removed under a dissecting microscope and separated individually at the mainstem bronchus. These studies were reviewed and approved by the institutional animal care and use committee (IACUC) at Duke University and the independent IACUC at the Durham Veteran Affairs Medical Center (VAMC).

**Organ culture.** The explant culture system was adapted from that of Chen and Little (2). Two to four lungs were removed and placed in DMEM (Gibco) with 5% fetal bovine serum added. Lungs were contained in 48-well tissue culture plates placed in humidified incubators at 37°C with an atmosphere of air plus 5% CO\textsubscript{2}. Ambient air in 5% CO\textsubscript{2} resulted in an inspired O\textsubscript{2} concentration of 18.5% (FICO\textsubscript{2} of 0.185) within the incubator, as measured with a fuel cell (Precision Instruments).

The lungs in organ culture were photographed daily on an inverted microscope (Nikon). The number of branches was determined from the resultant micrographs. We counted as branched all those terminal structures with an invagination sufficient to produce a pair of separate buds with a radius equal to the radius of the subtending airway. The fold increase in branching was calculated as the ratio of buds present at 72 h minus the buds present at 1 h, divided by the number of buds present at 1 h. Because of the variability in the bioassays, we routinely conducted concurrent control experiments with each experimental group.

**Chemicals.** NO donors were obtained from Alexis and were used in the final concentrations described in RESULTS. The NO donors were members of a class of diaziridines that donate their bound NO at 37°C, pH 7.4, within 5 min (Z)-1-(N-methyl-N-[6-(N-methyl-ammoniohexyl)amino])-diazen-1-ium-1,2-diolate NO (MAHMA-NO), 15 min (Z)-1-[N-(3-ammoniopropyl)-N-(n-propyl)-amino]-diazen-1-ium-1,2-diolate NO (PAPA-NO), or 20 h (Z)-1-[2-(2-aminooethyl)-N-(2-ammonioethy)l]amino]-diazen-1-ium-1,2-diolate NO (DETA-NO) (16). Each sterile addition of NO donor was made on a daily basis without changing the culture media. For the experiments in which depleted DETA-NO was used, we allowed the stock solution of DETA-NO to remain at 4°C and pH 7.4 for at least 5 days before its addition to the stock solution of DETA-NO to remain at 4°C and pH 7.4 for at least 5 days before its addition to the

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NO AND BRANCHING MORPHOGENESIS IN FETAL RAT LUNGS

with a commercial dye kit (Coomassie Plus, Pierce). Polya
crylamide gels were cast in a minigel holder (Hoefer) as
7.5% running gels with 2.5% stacking gels. Protein extracts
were centrifuged at 850 × average gravitational force (g_{avg})
for 10 min, and an aliquot of the supernate was denatured in
3% SDS according to Laemmli (12). Proteins were loaded
onto the stacking gel at 10–40 μg per lane. The gels were
electrophoresed at 30 mA until the dye front reached the
bottom of the gel. The gel was removed and placed in contact
with a nitrocellulose (Schleicher & Schuell) or polyvinylidene
difluoride (Immobilon, Millipore) membrane, and transfer of
the proteins was accomplished from 2 h to overnight, accord-
ing to Towbin et al. (25). Gels were checked for completeness
of the protein transfer by staining with Coomassie blue.

Immunoblots were first incubated in a blocking solution of
5% nonfat dry milk in Tris-buffered saline with 0.1% Tween
(TBST) for 1 h at room temperature. The membranes were
then incubated in primary antibody at dilutions of 1:1,000
(NOS III) or 1:500 (hypoxia-inducible factor-1α) in blocking
solution in sealed plastic bags overnight at 4°C. The mem-
branes were washed in TBST 3× for 10 min at room tempera-
ture. Secondary antibody (goat antimouse IgG conjugated
with horseradish peroxidase, Jackson Laboratories) was
used at 1:5,000–1:8,000 dilution for 60 min. After the mem-
branes were washed three times in large volumes of TBST for
at least 30 min, they were washed in TBS 1× for at least 15
min. The ECL chemiluminescence kit (Amersham) was em-
ployed as suggested by the manufacturer, and the blots were
placed in contact with photographic film (Biomax MR Kodak)
for 30 s–30 min before photographic development.

Statistics. Differences between groups were first tested by
ANOVA using a commercial software package (Prism, Graph-
Pad). For comparison of multiple means to a single control,
we used Dunnett’s test. Significance was set at P < 0.05.

RESULTS

Figure 1 is a representative example of the branch-
ing of gd 13 fetal rat lungs grown in DMEM in equi-
librium with ambient air and without (control) or with
100 μM DETA-NO added each 24 h. We found a dose-
dependent increase in branching at 50 and 100 μM
DETA-NO added (Fig. 2). Doses higher than 100 μM

 explant culture. The release of NO into the media was mea-
sured as nitrite with the Griess reaction (21).

N^G-monomethyl-l-arginine (l-NMMA), N^G-nitro-l-argi-
nine methyl ester (l-NAME), and 8-bromo-cGMP were
acquired from Calbiochem. Monoclonal antibodies (mAb) to
NOS III were acquired from Transduction Laboratories (no.
N30020).

Immunocytochemistry. After fixation in 4% paraformalde-
yde, tissue samples were embedded in paraffin and sec-
tioned on a microtome. Sections were mounted on glass slides
and deparaffinated in xylene. After rehydration, sections
were incubated in blocking solution, which consisted of 5%
normal goat serum, 5% nonfat dry milk, and 1% BSA in PBS,
for 30 min at room temperature. The sections were then
incubated with primary antibody at a concentration of 0.025
μg/μl of mAb or nonimmune rabbit IgG in a solution of 1%
nonfat dry milk and 1% BSA in PBS overnight at 4°C. Sec-
ondary antibody was rabbit anti-mouse IgG with bound
horseradish peroxidase (Jackson Laboratories) and was used
at concentrations of 1:500–1:2,000 diluted in 1% nonfat dry
milk and 1% BSA in PBS for 60 min at room temperature.
Color development was done using a diaminobenzidine reac-
tion kit (Turbo DAB, Innovex). Sections were counterstained
in 0.1% methylene blue for 10–30 s.

Immunoblotting. Proteins were extracted from tissues that
were homogenized (Polytron, Brinkmann) in a mixture of
solubilizing agent and antiproteases that included 0.2 M
cyclohexylaminopropanesulfonic acid, 2 mM EDTA, 4-[2-amino-
ethyl]benzenesulfonyl fluoride, aprotinin, bestatin, l-trans-
3-carboxyoxiran-2-carbonyl-l-leucylgmatine, leupeptin,
and pepstatin (Sigma). Protein concentrations were measured

Fig. 1. Branching of fetal rat lung explants was increased when
(Z)-1-[2-(2-aminoethyl)-N-(2-ammonioethyl)amino]-diazen-1-ium-1,2-
diolate nitric oxide (DETA-NO) was added to the culture media at
concentration of 100 μM. Control: gestational day (gd) 13 fetal lung
explant at 0 h (A) and after 72 h (B) in culture without added NO donor.
DETA-NO: gd 13 fetal lung explant at 0 h (C) and after 72 h (D) in
culture with added DETA-NO at 100 μM.

Fig. 2. Graphical summary of effect of adding the NO donor
DETA-NO to cultures of fetal rat lung. At 50 and 100 μM DETA-NO
added, there was a significant increase in branching compared with
control (no added DETA-NO). At greater additions of DETA-NO, a
progressive reduction in branching enhancement was found. At the
highest doses, no stimulation was found but there was evidence of
cellular damage (data not shown). Means ± SE. *P < 0.05 compared
with control (CONT).
resulted in fewer branches, and the morphology of those explants showed deterioration during the culture period. DETA-NO exhausted of its NO had no effect on branching (data not shown). Those NO donors with short half-lives, MAHMA-NO and PAPA-NO, had no effect on branching at the same doses that produced maximal effects with DETA-NO. The stoichiometry of NO released was measured as nitrite (NO$_2$) in the Griess reaction. There was a good quantitative relationship between the measured nitrite and the amount expected from complete release of bound NO from DETA-NO (92% ± 3% SE).

To investigate if DETA-NO increased airway branching via a cGMP-dependent pathway, up to 3-mM additions of 8-bromo-cGMP were made to the explant cultures. The addition of that cell-permeable and phosphatase-resistant analog of cGMP did not result in a change in the number of branches at 72 h compared with control lungs (Fig. 3).

The expression of NOS III protein in fetal and newborn rat lungs was evaluated by immunocytochemistry and by immunoblotting, shown in Figs. 4 and 5. NOS III was found in the endothelium of the large and small pulmonary vessels. The specificity of the mAb to NOS III is shown in the immunoblot of Fig. 4C. The ontogeny of NOS III protein expression in rat lung (Fig. 5) included a rise in NOS III protein through late gestation followed by a decline during postnatal lung development; NOS III was detected in the lungs of adult rats.

When compared with an age-matched fetus, the expression of lung immunoreactive NOS III protein in the cultured explants was reduced (Fig. 6). During the 72 h of culture, the NOS III protein increase was similar to the increase seen in vivo, although NOS III protein was lower in the explants compared with age-matched control lung tissue. We were unable to measure the metabolites of endogenously produced NO in the culture media using the Griess assay. This was due in part to significant background NO$_2$, which may have come from the serum supplement. The undetectable NO$_2$ accumulation in the media may also indicate that the rate of NO production by fetal lung explants was small.

As a test of the importance of endogenous NOS activity, lung explants were grown in the presence of two nonspecific but effective NOS inhibitors, L-NMMA and L-NAME. As shown in Fig. 7, the results of NOS inhibition were dependent upon the inhibitor. Fetal lungs grown in the presence of millimolar concentrations of L-NAME branched at a lower rate compared with the control explants. Lungs grown in ambient air

![Fig. 3. Effect of adding 8-bromo-cyclic guanine monophosphate (8-bromo) to the lung explant culture media. CONT, no 8-bromo added; 8-bromo, 1–3 mM concentrations of 8-bromo-cGMP added. Means ± SE. P > 0.05.](http://ajplung.physiology.org/)

![Fig. 4. Nitric oxide synthase (NOS) III expression in fetal rat lung on gd 16. A: control section with primary antibody omitted and nonimmune IgG added. B: NOS III was strongly expressed in large vessels (thick arrow) and small vessels (narrow arrows). C: immunoblot of gd 16 lung extract (20 µg/lane) as detected by the monoclonal antibody. A single band was found at ~130 kDa consistent with NOS III. mw, Molecular mass markers (in kDa).](http://ajplung.physiology.org/)
conditions plus millimolar concentrations of \( L\text{-NMMA} \) branched less than control lungs.

**DISCUSSION**

The time-dependent expression of NOS(s) in developing lung suggests that NO may regulate some event(s) during lung development. The physiological and clinical importance of lung NO production seems established for the purpose of lowering of pulmonary vascular resistance, which normally occurs at birth. The utility of NO therapy to the prematurely newborn human for the treatment of persistent pulmonary hypertension is consistent with NOS activity being a key developmental milestone. Any impact of NO production on morphological development is less clear. Reports of abnormal tracheal development and survival in *Drosophila* (26) and abnormal alveolar development in the fawn-hooded rat (13), in the presence of hypoxia, support the hypothesis that NO metabolism may provide an important modifying influence on lung development.

No systematic study in mammals of the influence of NO or of various \( O_2 \) atmospheres upon lung branching morphogenesis has been reported to our knowledge, although NO has been shown to mediate the hypoxia-accelerated development of the tracheal system in *Drosophila* (26). The model we used permitted direct observation of lung branching in vitro and allowed for defined additions to the incubation media. The branching pattern that occurs in this model only approximates that which occurs in vivo, but it has been useful for testing the potential importance of modulators of branching such as growth factors (18), matrix molecules including laminin (22) and tenascin (29), and for tissue recombination experiments (24). Because branching in the explant model is not precisely that of the lung in situ, any effect produced in cultured tissue may not translate in a simple way to development of the native lung. These experiments were performed on lungs separated from the trachea and primary bronchus, and thus any effect of NO on those structures is not included in this report. The viability of fetal lung tissue in culture was intact, as assessed by phase contrast microscopy showing adherence of the explant to the tissue culture plates, sharp cellular membranes, growth and branching in most conditions, and a normal-appearing cellular morphology in light microscopic sections. Only at the 500-\( \mu \text{M} \) concentration of DETA-NO was there evidence of cellular disruption consistent with toxicity. No metabolic tests of tissue viability were performed.

Figure 2 shows the dose-response curve of the effect of exogenous NO upon rat lung explant branching. Peak differences of greater than a twofold increase over control branching were observed at 100 \( \mu \text{M} \) of the

![Figure 5](image5.png)

**Fig. 5.** Ontogeny of NOS III expression in fetal and adult rat lung. Representative immunoblot loaded with 10 \( \mu \text{g} \) total protein/lane. The highest expression was found in late gestational fetal lung, and there was a decrease after birth. Immunoreactive NOS III was detected in adult lung but at a reduced concentration. Lane markers: mw, molecular mass; 1 = 13 gd lung; 2, 16 gd lung; 3, 20 gd lung; 4, postnatal day 4; 5, postnatal day 7; 6, postnatal day 14; 7, adult.

![Figure 6](image6.png)

**Fig. 6.** Immunoblot of NOS III expression in gd 13 and gd 16 rat lung and in gd 13 rat lung in explant culture for 24 and 72 h. There was less immunoreactive NOS III protein found in the cultured lung; both the cultured and the native lungs showed an increase in NOS III expression between gd 13 and gd 16 or its equivalent time point in the cultured lung. Twenty micrograms total lung protein were loaded per lane. Lanes: 1, gd 13; 2, gd 16; 3, gd 13 lung cultured for 24 h; 4, gd 13 lung cultured for 48 h in the presence of 100 \( \mu \text{M} \) DETA-NO; 5, gd 13 lung cultured for 72 h; and 6, gd 13 lung cultured for 72 h in the presence of 100 \( \mu \text{M} \) DETA-NO.

![Figure 7](image7.png)

**Fig. 7.** Effect of inhibition of endogenous NOS activity. Explants were cultured in an 95% ambient air-5% \( \text{CO}_2 \) atmosphere. NOS inhibitor was added at millimolar concentration at the beginning of the culture period. Means \( \pm \) SE. LNMMA, \( N^\text{G}\)-monomethyl-L-arginine; LNAME, \( N^\text{G}\)-nitro-L-arginine methyl ester. \(* P < 0.05 \) compared with control.
NO donor (DETA-NO), but significant differences compared with control were not found at higher levels. Morphological evaluation of the explants at the higher levels of DETA-NO suggested injury was occurring, because these explants occasionally detached from the culture dish, and refractile cytosolic granules were observed (data not shown). The increased branching produced by DETA-NO was evident on inspection, and the pattern of branching was not discernibly different from the control explants (Fig. 1). The NO-depleted donor had no effect on branching of the cultured lungs.

Other NONO-ates were studied to evaluate whether the effects were due to the rate of NO generation or to the total dose of NO. The shorter-acting MAHMA-NO (t$_{1/2}$ = 1 min at 37°C) and PAPA-NO (t$_{1/2}$ = 15 min at 37°C) had no effect on branching when used at 100-μM concentrations. Because of their rapid release of NO and the short half-life of the gas in aqueous medium, a much higher peak level of NO and a much shorter duration of the NO would occur. Neither of the short-acting donors had an effect.

Many effects of NO are mediated through activation of guanyl cyclase and a resultant increase in cellular cGMP. In the simple tracheal system of Drosophila, modulation of airway development by hypoxia was mediated by the cGMP pathway of NO (26). We tested this possibility in fetal rat lung growth with up to 3-mM concentrations of the phosphatase-resistant, cell-permeable 8-bromo-cGMP. No effects were seen (Fig. 3). This suggests the increased airway branching in response to DETA-NO was not due to effects mediated via cGMP. NO is capable of many interactions, including protein nitrosylation, the oxidation of thiols, and effects on Fe-or Fe-S-containing proteins other than guanyl cyclase, and these events could be involved in producing the effects we observed on rat lung branching.

Inhibition of endogenous NOS with two nonspecific enzyme inhibitors, L-NMMA and L-NNAME, was tested to determine if branching was influenced by endogenous NO production in the explants. In experiments conducted in ambient air, both NOS inhibitors reduced branching, although only L-NNAME was statistically effective. Under lowered oxygen tension, L-NMMA addition resulted in a statistically significant reduction in branching (data not shown).

The modest effects of NOS inhibitors to decrease airway branching in explants may suggest that endogenously produced NO has no physiological role in lung morphogenesis. We compared the amount of NOS III antigen in immunoblots loaded with equal amounts of homogenate protein from freshly harvested fetal lungs and from cultured lung explants matched for relative gestational age. After 24 h in culture, there was a lower amount of NOS III protein in the explant. If this resulted in a relative deficiency of NO production, then the explant might respond to exogenous NO with an increased level of branching as we observed. A more definitive test of the role of endogenous NO production during in vivo development of the lung may be better done with the use of the NOS III knockout mouse. As adults, both homozygous and heterozygous mice had an abnormal pulmonary vascular response to mild hypoxia (6). No details of lung morphology during development of these mice have been published.

The mechanism of any effect of NO on lung development is currently unknown, and the pleiotropic effects of NO in various systems makes difficult the prediction of its effects in a given circumstance. One expected effect of NO would be to enhance or inhibit apoptosis, and under many circumstances, NO acts to inhibit apoptosis (23). The rate and location of any selective apoptosis of airway cells could play a role in guiding branching. A second mechanism by which NO could modulate branching would be through direct toxic effects of RNS. High concentrations of NO would likely result in excess RNS, including some, like ONOO$^-$, capable of causing cellular damage. Production rates of NO by the constitutively expressed calcium-dependent NOSs are expected to result in nanomolar to about 1-μM tissue concentrations (27). The media concentrations of NO produced by our DETA-NO additions would be expected to be nearly micromolar (A. Gow, personal communication). The biphasic dose-response stimulation of branching in the explanted lung could be expected as RNS formation might begin to dominate the signaling effects of low NO concentrations.

The studies reported herein demonstrate the potential for NO to influence lung development at the branching morphogenesis stage, although we have not proven that NO is necessary nor sufficient for the branching events that occur in vivo. The physiological role for NO during normal development remains largely unknown.

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