Nitric oxide synthase isoform expression in the developing lung epithelium

TODD S. SHERMAN,1 ZHONG CHEN,1 IVAN S. YUHANNA,1 KIM S. LAU,2 LINDA R. MARGRAF,3 AND PHILIP W. SHAUL1

Departments of 1Pediatrics, 2Physiology, and 3Pathology, University of Texas Southwestern Medical Center at Dallas, Dallas, Texas 75235

Sherman, Todd S., Zhong Chen, Ivan S. Yuhanna, Kim S. Lau, Linda R. Margraf, and Philip W. Shaul. Nitric oxide synthase isoform expression in the developing lung epithelium. Am. J. Physiol. 276 (Lung Cell. Mol. Physiol. 20): L383–L390, 1999.—Nitric oxide (NO), generated by NO synthase (NOS), is an important mediator of physiological processes in the airway and lung parenchyma, and there is evidence that the pulmonary expression of the endothelial isoform of NOS (eNOS) is developmentally regulated. The purpose of the present study was to delineate the cellular distribution of expression of eNOS in the developing respiratory epithelium and to compare it with inducible (iNOS) and neuronal (nNOS) NOS. Immunohistochemistry was performed on fetal (125–135 days gestation, term 144 days), newborn (2–4 wk), and maternal sheep lungs. In fetal lung, eNOS expression was evident in bronchial and proximal bronchiolar epithelium but was absent in terminal and respiratory bronchiolae and alveolar epithelium. Similar to eNOS, iNOS was detected in bronchial and proximal bronchiolar epithelium but not in alveolar epithelium. However, iNOS was also detected in terminal and respiratory bronchiolae. nNOS was found in epithelium at all levels including the alveolar wall. iNOS and nNOS were also detected in airway and vascular smooth muscle. The cellular distribution of all three isoforms was similar in fetal, newborn, and adult lungs. Findings in the epithelium were confirmed by isoform-specific reverse transcription-polymerase chain reaction assays and NADPH diaphorase histochemistry. Thus the three NOS isoforms are commonly expressed in proximal lung epithelium and are differentially expressed in distal lung epithelium. All three isoforms may be important sources of epithelium-derived NO throughout lung development.

airway; inducible nitric oxide synthase; reduced nicotinamide adenine dinucleotide phosphate diaphorase; neuronal nitric oxide synthase; smooth muscle

There is accumulating evidence that NO is of great importance to lung epithelial function in the perinatal period. It has been shown that acetylcholine and bradykinin, which stimulate NO synthesis, cause marked decreases in lung liquid production in late-gestation fetal lambs (6). In addition, the instillation of NO or cGMP, the second messenger for NO, directly into the fetal lung liquid has the same effect (7, 8). The decrease in lung liquid production, which occurs in the respiratory epithelium at the time of birth, is an essential component of the transition of the fetus from liquid breathing to air breathing. Epithelium-derived NO is also critical to the regulation of bronchomotor tone in the developing lung, playing a key role in the opposition of airway contraction, particularly in the early newborn period (15). Furthermore, the pharmacological inhibition of NO production increases tissue resistance in the newborn lung, suggesting that endogenous NO may regulate peripheral contractile elements (21).

North et al. (20) and others (25) demonstrated that expression of the endothelial isoform of NOS (eNOS) in the lung increases during late gestation to be maximal near term, thereby optimizing the capacity for pulmonary NO production around the time of birth when this signaling molecule is required for lung liquid resorption and bronchodilation as well as for pulmonary vasodilation (25). Shaul et al. (26) and others (12) also demonstrated that eNOS is expressed in mature airway epithelium as well as in pulmonary endothelium. However, it is not yet known whether eNOS is expressed in fetal pulmonary epithelium or how eNOS is compared with epithelial eNOS expression with that of inducible (iNOS) and neuronal (nNOS) NOS, which are also known to be expressed in fetal lung (20, 22, 23, 31). Immunohistochemistry was employed to evaluate the cellular distribution of the three NOS isoforms. Their expression in airway epithelium was also assessed with reverse transcription-polymerase chain reaction (RT-PCR) assays and NADPH diaphorase histochemistry.

MATERIALS AND METHODS

Animal model. Lung tissue was studied from three groups of mixed-breed lambs. They were fetal lambs at 125–135 days gestation (term 144 ± 4 days), newborn lambs at 2–4 wk of age, and maternal sheep. The pregnant ewes used were

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multiparous and had singletons or twins. Before being killed, the animals were housed in the Animal Resources Center of the University of Texas Southwestern Medical Center at Dallas and given standard animal chow and water ad libitum. The ewes and fetus(es) were euthanized with pentobarbital sodium (120 mg/kg) given intravenously to the ewe, and the fetuses were delivered by cesarean section. The newborn lambs were killed in a similar manner. Both proximal and distal lung tissues were immediately removed and either immersed in 2% paraformaldehyde to await further processing for immunohistochemistry or frozen in liquid nitrogen for later NADPH diaphorase histochemistry. The procedures followed in the care and euthanasia of the study animals were approved by the Institutional Review Board for Animal Research.

Immunohistochemistry. Lung tissue was fixed in 2% paraformaldehyde in PBS for 4 h (4°C), immersed in an increasing sucrose-PBS gradient (10% sucrose for 90 min, 15% sucrose for 60 min, and 20% sucrose for 60 min at 4°C), fixed further for 2 h in 10% neutral buffered Formalin (Richard-Allan Scientific, Kalamazoo, MI) at 40°C, processed through graded alcohols, and embedded in paraffin. Four-micrometer deparaffinized sections were incubated for 18 h with primary antisera specific for either eNOS at a 1:100 dilution (mouse monoclonal antibody against human eNOS; Transduction Laboratories, Lexington, KY), iNOS at a 1:1,500 dilution (rabbit polyclonal antibody against murine iNOS), or nNOS at a 1:3,000 dilution (rabbit polyclonal antibody against rat nNOS; both from Upstate Biotechnology, Lake Placid, NY) at 4°C. In studies of eNOS and iNOS, the incubation with primary antisera was preceded by heat-induced epitope retrieval in basic buffer (AR10 buffer, Biogenex, San Ramon, CA). After endogenous peroxidases were quenched with 3% hydrogen peroxide in H2O2, immunostaining was performed with standard streptavidin-biotin-horseradish peroxidase detection methodology and hematoxylin counterstaining. All three NOS isoforms were evaluated simultaneously along with negative controls consisting of lung tissue incubated in the absence of primary antisera. Immunostaining of ovine fetal pulmonary arterial endothelial cells, lipopolysaccharide-treated bovine trachealis, and ovine cerebellum provided positive controls for eNOS, iNOS, and nNOS, respectively. Preparation of the bovine trachealis involved culturing individual strips (0.8 × 3.0 × 0.5 mm) in 1 ml of DMEM with 10 µg/ml of lipopolysaccharide (serotype 0127:B8, Sigma, St. Louis, MO) for 24 h. Findings were confirmed in at least three independent experiments in which all three age groups were immunostained simultaneously. NOS immunostaining was examined along the entire respiratory tree from bronchial to alveolar levels. With the use of standard definitions, bronchi were designated as airways associated with smooth muscle and cartilage, whereas bronchioles were lined with smooth muscle but not cartilage. Terminal bronchioles lacked both smooth muscle and cartilage and were lined by contiguous low-columnar to cuboidal epithelial cells, whereas respiratory bronchioles were partially lined by both cuboidal epithelial and respiratory alveolar type I and type II epithelial cells (28).

RT-PCR assays. To determine whether eNOS mRNA is expressed in fetal airway epithelial cells, RT-PCR assays were performed. The epithelial cell layer of main stem bronchi from fetal sheep was microdissected on ice with sterile techniques. The identity of the cell layer removed was confirmed histologically with hematoxylin and eosin staining of the bronchi (3). Total cellular RNA was obtained from the epithelial cells by a single extraction with an acid guanidinium thiocyanate-phenol-chloroform mixture (5). RT was performed with 5 µg of total RNA by methods previously reported (20). Briefly, cDNA synthesis was carried out with 200 U of Moloney murine leukemia virus reverse transcriptase, 5 µM oligo(dT), 1 mM deoxynucleotide triphosphates and 3 mM Mg2+ in a volume of 20 µl. In selected tubes, the reverse transcriptase was omitted to control for amplification from contaminating cDNA or genomic DNA. The temperature profile was 1) annealing at room temperature for 5 min, 2) extension at 42°C for 60 min, and 3) termination at 99°C for 5 min.

PCR was performed on the resulting RT product with specific oligonucleotide primers designed from the sequence for sheep eNOS cDNA (27). The sequence of the sense primer was 5′-AGTCGAGACCCTCGAGTCAGGA-3′, and that of the antisense primer was 5′-GTCTCAAGCTTTGAGTGGC-3′. The PCRs contained 1.5 mM Mg2+, 1 µM primers, 200 µM
deoxynucleotide triphosphates, 20 mM Tris (pH 8.4), 50 mM KCl, and 5 µl of cDNA in a final volume of 50 µl. To minimize nonspecific amplification, a "hot-start" procedure was employed in which the PCR tubes were placed in a thermal cycler (Perkin-Elmer model 9600) prewarmed to 94°C. After 2 min, each tube was opened sequentially, and 2.5 U (in 2 µl) of Taq DNA polymerase were added. The PCR temperature profile consisted of 35 cycles at 94°C for 45 s (denaturation), 60°C for 45 s (annealing), and 72°C for 1.25 min (extension), followed by an additional 5-min final extension at 72°C. The primer location, primer concentration, Mg²⁺ concentration, and annealing temperature were optimized to produce the greatest amount of a single PCR product.

The PCR products were size fractionated by agarose gel electrophoresis, and product identity was confirmed by transferring the DNA to nylon filters and probing with a 32P-labeled internal oligonucleotide specific for eNOS (27). PCR product identity was also confirmed by direct double-stranded sequencing. Results were confirmed with three different total RNA samples.

To determine whether iNOS and nNOS mRNAs were also expressed in the airway epithelial cells, RT-PCR assays for these isoforms were done in a similar manner. PCR for iNOS employed primers designed from the murine iNOS cDNA (18), and PCR for nNOS used primers designed from the rat nNOS cDNA (20). The primer pair for iNOS was 5'-AAATCCTACCAGTGACCT-3' (sense) and 5'-AGACTGGACTTTGCAATGTA-3' (antisense), and the primer pair for nNOS was 5'-GAATACAGCTGATCCATGGAAC-3' (sense) and 5'-TCCTCCAGGGGTGTCACC CGCATG-3' (antisense). For iNOS, the PCR temperature profile consisted of 35 cycles at 94°C for 20 s (denaturation), 54°C for 30 s (annealing), and 72°C for 30 s (extension), followed by an additional 5-min final extension at 72°C. The PCR temperature profile for nNOS was identical to that described above for eNOS.

NADPH diaphorase histochemistry. To confirm the findings obtained with immunohistochemistry and RT-PCR, NADPH diaphorase histochemistry was performed, which will detect the activity of any NOS isoform (31). Lung tissue stored in liquid nitrogen was briefly thawed at room temperature, embedded in optimum cutting temperature compound (Sakura, Tokyo, Japan), cryostat sectioned at −20°C, and thaw mounted on positive-charged slides. Cryosections were fixed in freshly prepared 2% paraformaldehyde in PBS for 10 min at 4°C, rinsed in PBS, and incubated with 1 mg/ml of β-NADPH, 0.5% Triton X-100, and 0.25 mg/ml of nitro blue tetrazolium in 0.1 M Tris buffer, pH 8.0, for 1 h at room temperature in a dark, humidified chamber. Negative controls were incubated without the β-NADPH substrate. After being rinsed in PBS, the sections were counterstained with methyl green, coated with aqueous mounting medium (Crystal/Mount, Biomeda, Foster City, CA), heated for 10 min at 70°C, postmounted with organic mounting medium (Permount, Fisher Scientific, Pittsburgh, PA), and sealed with coverslips.

RESULTS

eNOS immunohistochemistry. The distribution of eNOS in the fetal respiratory epithelium is shown in Fig. 1. Positive immunostaining for eNOS was observed in bronchial and proximal bronchiolar epithelia (Fig. 1,
A and B, respectively). However, the signal for eNOS was not found in epithelium at the terminal bronchial or alveolar level (Fig. 1C). As expected, eNOS immunostaining was readily seen in vascular endothelial cells at all levels. In the negative control for all three NOS isoforms (Fig. 1D), there was a complete absence of immunostaining in the airway epithelium, the vascular endothelium, and all other cell types.

To determine whether there were maturational changes in the epithelial distribution of eNOS expression, simultaneous immunohistochemical studies were done in fetal, newborn, and adult sheep lungs. Findings at the bronchiolar level are shown in Fig. 2. In all three age groups, positive eNOS immunostaining was visible in airway epithelial cells. Similar to the distribution of epithelial cell staining in the fetal lung, eNOS was present in newborn and adult bronchial and bronchiolar epithelia but was absent in terminal and respiratory bronchioles and alveolar epithelium from these two age groups (data not shown).

iNOS immunohistochemistry. The distribution of iNOS in the fetal respiratory epithelium is depicted in Fig. 3. Similar to eNOS, positive iNOS immunostaining for iNOS was observed in the airway epithelial cells at the level of the bronchus and proximal bronchiole (Fig. 3A and B, respectively). However, in contrast to eNOS, iNOS was also detected in the epithelium of terminal and respiratory bronchioles (Fig. 3C). iNOS immunostaining was not evident in the alveolar wall. iNOS was detected in modest amounts in both airway and vascular smooth muscle (Fig. 3A). No staining was observed in vascular endothelial cells.

The ontogeny of epithelial iNOS immunostaining at the level of the bronchiole is shown in Fig. 4. iNOS was noted in the airway epithelium of all three age groups. The distribution of iNOS expression in the epithelia of the bronchus, proximal bronchiole, and terminal and respiratory bronchioles was constant in all age groups (data not shown). Staining of airway epithelium and vascular smooth muscle was greatest in the fetal lung, whereas airway smooth muscle staining was greatest in the maternal lung.

nNOS immunohistochemistry. The distribution of nNOS in the fetal respiratory epithelium is shown in Fig. 5. Intense nNOS immunostaining was seen in airway epithelial cells associated with the bronchus (Fig. 5A), the proximal bronchiole (Fig. 5B), and the terminal and respiratory bronchioles (Fig. 5C). In contrast to both eNOS and iNOS, alveolar epithelial cells also displayed nNOS staining (Fig. 5C). Both airway and vascular smooth muscle staining was similarly robust (Fig. 5A). There was an absence of nNOS staining in the vascular endothelium.

The ontogeny of nNOS immunostaining at the level of the bronchiole is shown in Fig. 6. Intense immunostaining for nNOS was seen throughout the respiratory
tree, from bronchus to alveolar wall in all age groups. Positive staining of the airway smooth muscle and vascular smooth muscle was also evident at all ages.

RT-PCR assays. To determine whether eNOS mRNA was expressed in the fetal airway epithelium, RT-PCR was performed with total RNA isolated from microdissected fetal bronchial epithelial cells with primers specific for sheep eNOS (Fig. 7, top). The assay was done in the presence and absence of the reverse transcriptase enzyme to assess any potential false signal from contaminating cDNA or genomic DNA. A representative Southern blot is shown. A single RT-PCR product of the correct predicted size of 281 bp was obtained only in the presence of reverse transcriptase, and its identity was confirmed by both Southern analysis with an internal oligonucleotide and direct sequencing. Similar studies were performed for iNOS and nNOS, yielding RT-PCR products of the correct predicted sizes of 291 and 602 bp, respectively (Fig. 7, middle and bottom, respectively).

NADPH diaphorase histochemistry. NADPH diaphorase histochemistry was employed to confirm NOS expression in all the cell types studied. Positive and negative control studies were performed and are displayed for the fetal airway (Fig. 8, A and B, respectively), vasculature (Fig. 8, C and D, respectively), and alveolar wall (Fig. 8, E and F, respectively). The deep blue stain indicates NADPH diaphorase activity, whereas methyl green counterstains the nuclei. Diaphorase activity was readily visible in both airway epithelial cells and airway smooth muscle (Fig. 8A) and in both vascular endothelial cells and vascular smooth muscle (Fig. 8C). Diaphorase activity was also evident in alveolar epithelium (Fig. 8E). Similar findings were obtained in newborn and adult lungs. These findings suggest that eNOS is a critical source of airway NO throughout fetal and postnatal development.

The observation that eNOS protein is expressed in mature proximal airway epithelium is consistent with studies in the adult human lung using both immunohis-
tochemistry and in situ hybridization (12) and in the adult rat lung using immunohistochemistry (30). It is also consistent with the previous identification by Shaul et al. (27) of constitutive eNOS expression in NCI-H441 cultured human bronchiolar epithelial cells by degenerate PCR. The present finding that eNOS is expressed in newborn proximal airway epithelium is also in agreement with the results of a study in the rat (30). However, our demonstration of eNOS protein expression in fetal airway epithelium contrasts with previous studies (14, 31) in both rats and sheep that did not detect eNOS in the fetal airway. The present observations using immunohistochemistry with an isoform-specific antiserum have been confirmed by RT-PCR, indicating that they are not due to cross-reactivity with either iNOS or nNOS. To our knowledge, this is the first evidence of epithelial eNOS expression in the fetal lung of any species.

Along with the studies of eNOS expression, experiments were performed to compare epithelial eNOS expression with that of iNOS and nNOS. Similar to eNOS, positive immunostaining for iNOS was observed in the airway epithelial cells at the level of the bronchus and proximal bronchiole, and it was not evident in the alveolar wall. However, in contrast to eNOS, iNOS was also detected in the epithelium of terminal and respiratory bronchioles. The distribution of iNOS expression in the epithelium of the bronchus, proximal bronchiole, and terminal and respiratory bronchioles was constant in all age groups. These findings indicate that iNOS is constitutively expressed in the normal lung in respiratory epithelial cells that also express eNOS, as well as in cells lacking eNOS. Constitutive iNOS expression has been previously demonstrated in the epithelium of adult and fetal rat tracheae and also in both fetal and adult human bronchial and bronchiolar epithelia (17, 28–30).

In studies specifically evaluating the distribution of epithelial nNOS, intense NOS immunostaining was seen in airway epithelial cells associated with the bronchus, the proximal bronchiole, and the terminal and respiratory bronchioles. However, in contrast to both eNOS and iNOS, alveolar epithelial cells also displayed NOS staining, and this pattern of expression was similar in fetal, newborn, and adult lungs. Studies of rat lung have previously demonstrated nNOS expression proximal to the alveolar ducts (17, 24, 31), but this is the first report of nNOS in the alveolar wall. Because the isoform-specific antisera to eNOS and iNOS did not reveal positive immunostaining in the alveolar wall, it is likely that NOS is the primary, if not the only, NOS isoform expressed in the distal lung epithelium. The expression of NOS in the alveolar epithelium is confirmed by the finding of positive

Fig. 8. NADPH diaphorase histochemistry in ovine fetal lung. Positive and negative control studies are shown for airway (A and B, respectively), vasculature (C and D, respectively), and alveolar wall (E and F, respectively). Diaphorase activity was visible in both airway epithelial cells (arrows) and airway smooth muscle, in both vascular endothelial cells (arrowheads) and vascular smooth muscle, and in alveolar epithelium.
NADPH diaphorase activity. Taken collectively, these observations reveal that whereas the three NOS isoforms are commonly expressed in the proximal lung epithelium, they are differentially expressed in the distal lung epithelium. A degree of caution is warranted, however, in directly equating the immunohistochemical findings with levels of enzyme activity because the NOS enzymes are regulated extensively at the posttranslational level (9).

In addition to comparing the expression of the three NOS isoforms in the respiratory epithelium of the fetal, newborn, and adult lungs, the present study has also revealed the nonepithelial cells that express eNOS, iNOS, or nNOS. As predicted, eNOS was detected in pulmonary endothelial cells. However, iNOS immunostaining was found in both airway and vascular smooth muscle cells, and the staining in the airway smooth muscle cells was greatest in the adult, whereas the staining in the vascular smooth muscle cells was most evident in the fetus. In addition, robust immunostaining for nNOS was seen in airway and vascular smooth muscle cells. To our knowledge, this is the first description of constitutive iNOS or nNOS expression in pulmonary vascular or airway smooth muscle cells.

The observed constitutive expression of all three isoforms in the proximal airway epithelium throughout development suggests that any of the three may play a role in the regulation of bronchomotor tone. In studies of isolated trachea from piglets, the nonspecific NOS inhibitor nitro-L-arginine methyl ester (L-NAME) caused an increase in the contractile response of the airway to acetylcholine, as did removal of the epithelium. The latter cell type is the likely primary source of endogenous NO because L-NAME no longer influenced the contractile response in the absence of the epithelium (15). Similarly, in studies of ventilated, open-chest newborn piglets, L-NAME caused an increase in whole lung resistance and also changes in airway resistance (21). Experiments with isoform-specific NOS antagonists or isoform-specific gene knockout models will be required to determine the relative contribution of each isoform to airway function.

There is also evidence of a role for NO in distal lung function, including the regulation of lung liquid production around the time of birth. The infusion of the NO agonists acetylcholine and bradykinin caused a marked decrease in lung liquid production in late-gestation fetal lambs (6), as did the instillation of NO or cGMP directly into the fetal lung liquid (7, 8). Lung liquid production represents a balance between Cl− secretion and Na+ reabsorption by the alveolar epithelium (16). The effect of NO to decrease lung liquid production may be through changes in Cl− permeability because a recent study (4) in cultured ovine fetal distal lung epithelium showed that NO causes a fall in Cl− transport. In addition, NO may be involved in the regulation of lung compliance. L-NAME administration causes a reduction in tissue resistance in newborn piglet lungs, suggesting a physiological role for endogenous NO in the regulation of peripheral contractile elements (21). Our present finding that nNOS is the principal isoform expressed in alveolar lining cells suggests that the neuronal isoform is important to the regulation of lung water balance and the elasticity of the distal lung parenchyma. Interestingly, North et al. (20) previously showed that the expression of nNOS in whole rat lung increases to be maximal near term and then declines postnatally. Once again, studies with isoform-specific NOS antagonists or isoform-specific gene knockout models will be needed to further evaluate the role of nNOS in the distal lung parenchyma during the perinatal period.

In addition to having functions in the developing airway and lung parenchyma, endogenous NO plays a key role in the regulation of pulmonary vasomotor tone in the perinatal period (25). This was originally revealed in a study (1) using nonspecific NOS antagonists such as nitro-L-arginine (1). However, more recent experiments have addressed the unique contributions of iNOS and nNOS to vascular regulation. In fetal lambs, the intrapulmonary infusion of three selective iNOS antagonists caused 69–82% increases in pulmonary vascular resistance (22). Similarly, it has recently been reported that the infusion of an nNOS-specific antagonist also increases pulmonary vascular resistance in the fetal lamb (23). These observations suggest that both iNOS and nNOS contribute to the modulation of vascular tone in the developing fetal lung. Based on the present findings, it is likely that the iNOS and nNOS involved in this process reside in the vascular smooth muscle. However, the airway epithelium and airway smooth muscle must also be considered as alternative sources of iNOS- and nNOS-derived NO. Site-directed modulation of the expression of these isoforms will be required to elucidate the exact cellular sources involved in pulmonary vascular control.

The present investigation has revealed that a high degree of complexity characterizes the expression of eNOS and also that of iNOS and nNOS in the developing respiratory epithelium. Future studies of the regulation of these individual isoforms in the developing lung will be needed to enhance our basic understanding of the role of NOS in epithelium of both pulmonary and nonpulmonary origin.

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Address for reprint requests: P. W. Shaul, Dept. of Pediatrics, Univ. of Texas Southwestern Medical Center, 5323 Harry Hines Blvd., Dallas, TX 75235-9063.

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