Pulmonary NO synthase expression is attenuated in a fetal baboon model of chronic lung disease

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The normal functions of NO in the mature airway include neurotransmission, smooth muscle relaxation, bacteriostasis, and the modulation of mucin secretion, ciliary motility, and plasma exudation (6, 16). Physiological actions of NO in the lung are most likely not mediated by free NO but rather by products of NO reactions, including S-nitrosocysteines and S-nitrosothiols (17).

There is accumulating evidence that NO is of major importance to lung epithelial function in the perinatal period. Epithelium-derived NO plays a key role in the opposition of airway contraction (19) in the modulation of lung liquid production (10) and in the regulation of peripheral contractile elements in the developing lung (19). In addition, NO has a well-recognized role in mediating pulmonary vasomotor tone in the perinatal period (33). In recent studies of lungs from fetal baboons, we have shown that all three NOS isoforms, neuronal NOS (nNOS), endothelial NOS (eNOS), and inducible NOS (iNOS), are primarily expressed in proximal respiratory epithelium in the developing primate. In addition, there are maturational increases in the expression of the three NOS isoforms and in NO production during the early third trimester that may contribute to enhanced airway and parenchymal function in the immediate postnatal period (34).

Along with serving a role in normal lung function, there is evidence that changes in pulmonary NO production related to altered NOS expression contribute to the cellular damage and parenchymal dysfunction characteristic of many pulmonary inflammatory conditions. In multiple paradigms of lung inflammation, iNOS is upregulated to produce cytotoxic levels of NO (30). However, despite iNOS upregulation, the net effect of inflammation can be an attenuation in epithelial NO production, such as in the guinea pig model of parainfluenza infection, leading to airway hyperresponsiveness (12). In a similar manner, lung transplant recipients with obliterative bronchiolitis display greater iNOS expression and attenuated nNOS and eNOS expression compared with controls (25). Thus...
there is evidence in the mature lung that, concomitant with NO overproduction by iNOS during pulmonary inflammation, which potentially contributes to cellular damage, NO production by nNOS and eNOS may be diminished, resulting in airway, parenchymal, and vascular dysfunction.

Chronic lung disease (CLD) is an inflammatory process that can be initiated following the early course of hyaline membrane disease in premature infants requiring ventilatory support. Before the surfactant treatment era, CLD was characterized by airway injury, parenchymal fibrosis, and inflammation. The more recent form of CLD is notable for less fibrosis and fewer and larger alveolae and an increase in elastic tissue that correlates with the severity of the clinical disorder (40). Infants with CLD have increased pulmonary vascular and airway resistance (1), and similar functional abnormalities have been documented in chronically ventilated preterm lambs and baboons (2, 7, 9, 45). In the preterm lamb model, there is also evidence that eNOS protein abundance is attenuated in small intrapulmonary arteries and small airways compared with those from control lambs born at term; in contrast, iNOS abundance is unchanged (23). Thus attenuated eNOS expression may play a role in the pathogenesis of CLD. However, it is not known whether such changes in eNOS abundance lead to alterations in pulmonary NO production or whether nNOS is affected, and it is also unclear whether similar mechanisms occur after preterm birth and ventilatory support in primes or higher species.

To better understand the potential role of pulmonary NOS in the genesis of CLD associated with preterm birth in humans, we evaluated lung NOS expression and function in a model of CLD in baboon fetuses delivered at 125 days of gestation (term = 185 d) and placed on ventilatory support for 14 d. The baboons are born at 67% of term gestation, which is comparable to 27 wk postconceptional age in humans, and the model closely mimics the current form of CLD in extremely preterm human infants (9, 45). Control samples were obtained from additional fetuses at 125 or 140 d of gestation. On the basis of the findings in other paradigms of lung inflammation (12, 25, 30), we tested the hypothesis that nNOS and eNOS expression are diminished and iNOS expression is upregulated in the baboon model of CLD. The specific contributions of each of the three NOS isoforms to enzymatic activity were also assessed. In addition, we determined whether changes in NOS isoform expression are associated with alterations in lung NO output evaluated by longitudinal measurements of the minute ventilation of NO at standard temperature and pressure (VeNOSTP).

MATERIALS AND METHODS

Animal model. All animal studies were performed at the Southwest Foundation for Biomedical Research Primate Center in San Antonio, TX. Pregnancies in baboons (Papio papio) were timed with cycle dates, and fetal growth parameters were obtained from prenatal ultrasound examinations performed at 70 and 100 d of estimated fetal gestation. Fetal baboons were delivered at 125 ± 2 d of gestation (term = 185 d) by cesarean section. At birth, the baboons were weighed, sedated, intubated, and given 4 ml/kg of surfactant (Survanta; courtesy of Ross Laboratories, Columbus, OH) before initiation of ventilator support. Ventilation was provided for 14 d with a humidified, pressure-limited, time-cycled infant ventilator (InfantStar; Infrasonics, San Diego, CA), and stabilization also included the placement of an umbilical arterial catheter and percutaneous central venous catheter. Details of animal care have been published elsewhere (9). Exhaled NO levels were measured at hour 1, 8, and 24 of life and daily from day 2–13 of life. At the time of necropsy on day 14 of life, proximal lung specimens were snap-frozen for later analysis. The samples were taken from the most proximal one-third of the lung parenchyma adjacent to the bronchus. Maturational control specimens were obtained at the time of hysterotomy from additional fetuses at 125 d of gestation (beginning of study) and 140 d of gestational age (end of study). Lung samples were obtained from five animals per group. NOS enzymatic activity was evaluated in n = 5 per group, and immunoblot analyses were described in n = 4 per group (36). Experiments were performed on an aliquot of tissue availability. Total lung wet weights were not changed in CLD animals at 14 days of life compared with 125-d gestation controls (11.1 ± 0.8 vs. 9.7 ± 0.5 g, respectively, P = not significant). In contrast, lung wet weights in 140-d gestation controls (12.6 ± 0.5 g) were greater than at 125 d of gestation (P < 0.05). VeNOSTP was assessed longitudinally in a total of eight CLD animals due to the availability of three additional animals in the consortium.

Ventilatory management. The ventilatory approach entailed a strategy to maintain tidal volumes at 4–6 ml/kg as determined with a body plethysmograph system (VT1000; Vital-trends Technology, New York, NY) and to generate adequate chest motion by clinical examination. Tidal volumes did not vary significantly over the course of the study. Initial positive end-expiratory pressure within the first 1–2 h of life was 4–5 cmH2O and it was weaned thereafter to a minimum of 3 cmH2O and was not significantly changed during the remaining course of study. Bias flow was maintained at 8 l/min. Target arterial blood gas parameters were PaCO2 45–55 Torr and PaO2 55–70 Torr. In an attempt to minimize exposure to high FIO2, if the PaO2 level was above target guidelines, a chest radiograph was obtained. If PaO2 was below target guidelines, a chest radiograph was obtained to evaluate lung inflation. Adjustments in mean airway pressure were made to minimize underinflation or overinflation of the lung. If lung inflation was deemed adequate, FiO2 alone was adjusted.

NOS enzymatic activity. Determinations of NOS enzymatic activity in the presence of excess substrate and cofactors provide a reliable quantitative assessment of enzyme abundance. As importantly, pharmacological interventions within the activity assay provide the only means to effectively assess the relative abundance of the three NOS isoforms and their individual contributions to total activity. NOS enzymatic activity was determined in proximal specimens of lung obtained at necropsy at 14 d of postnatal life in the baboons delivered at 125 d of gestation (CLD) and in the 125- and 140-d gestation maturational controls by previously described methods (36). Enzyme measurements were performed on the proximal one-third of lung parenchyma, which is rich in large airways. Although nonairway cell types were also included, selected dissection of airways was not feasible when multiple aliquots of tissue must be frozen immediately for use by the consortium. The lung tissue, which was initially snap-frozen
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and stored in liquid nitrogen, was slowly thawed and homogenized on ice in 50 mM Tris buffer (pH 7.8) containing 10 μg/ml pepstatin A, 10 μg/ml leupeptin, 10 μg/ml aprotinin, 10 μg/ml N-α-p-tosyl-L-lysine chloromethyl ketone, 20 μM tetrahydrobiopterin, 3.0 mM dithiothreitol, 1.0 mM phenylmethylsulfonyl fluoride, and 10 mM 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate using a ground glass homogenizer. The tissue homogenate was centrifuged at 10,000 g, total protein content was determined on the supernatant by the Bradford method using bovine serum albumin as the standard (8), and NOS activity was determined in the supernatant by measuring the conversion of [³H]-L-arginine to [³H]-L-citrulline (26). Tissue preparation (50 μl) was added to 50 μl of buffer, yielding final concentrations of reagents as follows: 2 mM β-NADPH, 2 μM tetrahydrobiopterin, 10 μM flavin adenine dinucleotide, 10 μM flavin mononucleotide, 0.5 mM CaCl₂ in excess of EDTA, 15 mM calmodulin, 2 μM cold L-arginine, and 2.0 μCi/ml [³H]-arginine. After incubation at 37°C for 30 min, the assay was terminated by the addition of 400 μl of 40 mM HEPES buffer, pH 5.5, with 2 mM EDTA and 2 mM EGTA. The terminated reaction was added to 1-ml columns of Dowex AG50WX-8 (Tris form) and eluted with 1 ml of the 40 mM HEPES buffer. Loading of NOS activity was evaluated by the addition of 2.5 mM EGTA to the incubation mixture. To partition the calcium-dependent NOS activity into that derived from nNOS vs. eNOS, we performed additional enzymatic activity assays in the absence and presence of the non-selective antagonist S-methyl-L-thiocitrulline (SMTCT, 10⁻⁸ M) (13). The isoform selectivity of SMTCT was first confirmed in dose-response studies of NOS enzymatic activity in lysates of COS-7 cells transiently transfected with cDNA for nNOS, eNOS, or iNOS by previously described approaches (35). SMTCT caused 88% inhibition of nNOS activity, and it had negligible effects on eNOS or iNOS activity.

Immunoblot analyses. Immunoblot analyses for nNOS, eNOS, and iNOS provide an additional independent means of quantifying differences in the expression of a given isoform between study groups. The methods for immunoblot analysis were similar to those previously reported (36). Proximal lung samples were thawed on ice and homogenized in ice-cold 50 mM Tris buffer and processed as described above for enzymatic activity analysis. SDS-PAGE was performed with 7% acrylamide, and the proteins were electrophoretically transferred to nitrocellulose filters. The filters were blocked for 1.5 h in buffer containing 150 mM NaCl and 10 mM Tris (pH 7.5) with 0.5% Tween 20 and 5% dried milk and incubated overnight at 4°C with polyclonal antibody generated either to the unique midmolecule peptide PYNSSPRQEGHKSYK of eNOS, which corresponds to a conserved epitope identical in sequence between mouse, canine, guinea pig, bovine, or human, or to the COOH-terminal peptide ESJKDITDVEFS of nNOS that is identical in sequence in mouse and human. iNOS protein abundance was quantitated using monoclonal antibody reactive to both the mouse and human protein (Transduction Laboratories, San Diego, CA). After incubation with primary antisera, the nitrocellulose filters were washed with the 150 mM NaCl buffer with Tween 20 and incubated for 1.5 h with donkey anti-rabbit Ig or rabbit anti-mouse Ig antibody-horseradish peroxidase conjugates (Amersham). The filters were washed in the 150 mM NaCl buffer with Tween 20, and the bands for NOS were visualized by chemiluminescence (ECL Western Blotting Analysis System; Amersham) and quantitated densitometrically. The antiserum to eNOS was the kind gift of Dr. Thomas Michel (Cardiovascular Division, Brigham and Women’s Hospital, Harvard Medical School, Boston, MA), and the antiserum to nNOS was the kind gift of Dr. Kim Lau (Department of Physiology, University of Texas Southwestern Medical Center).

Measurements of exhaled NO. The procedures employed were in accordance with the 1999 American Thoracic Society (ATS) recommendations (3, 38). A chemiluminescence analyzer (EcoPhysics CLD 77 AM; EcoPhysics, Duernten, Switzerland) was used to measure NO in exhaled breath. This device detects light produced by the reaction of ozone with NO using a charge-coupled digital detection system, with subsequent 10-Hz digital signal output. The analyzer performs an internal calibration by serial dilution of a known concentration NO gas standard (8 parts per million (ppm), National Institute of Standards and Technology (NIST) traceable cylinder of Environmental Protection Agency (EPA) reference grade; Scott Specialty Gasses, Plumsteadville, PA) using NO scrubbed (Purafil, Doraville, GA) zero gas as the diluent. The internal calibration was made by the software. The resulting signal were verified by comparison against serial dilutions of an external standard. Briefly, NO standard gas (10 ppm, NIST traceable cylinder of EPA reference grade; Scott Specialty Gasses) was diluted with air passed through an NO scavenging apparatus (Purafil) using NIST traceable mass flow controllers (Cole-Farmer, Vernon Hills, IL) to achieve five known concentrations of NO over the range of 2–80 parts per billion (ppb). The correlation coefficient (r) for measured vs. predicted values was 0.998. Response time of the device was determined by the diaphragm puncture technique (31, 32). The response time to 90% of full strength signal was <0.1 s (21).

The high ventilator rate employed in a low volume ventilation strategy hampers continuous sampling of exhaled NO from a distal endotracheal port. Additionally, single breath profiles obtained from nonintubated adult volunteers have demonstrated marked flow dependence for exhaled NO (37). We therefore devised a sampling strategy that maintains a constant flow rate and is rapid enough to minimize interference with the animal’s Vt yet long enough to ensure an adequately stable peak signal for analysis. A low-volume, non-flow-restricting dual stopcock was interposed between the endotracheal tube adaptor and the proximal endotracheal tube. In the baseline position the animal was continuously ventilated, while the analyzer sampled inspired gas from the inhalation limb of the ventilator circuit. At full inspiration (noted by chest rise), the stopcock was rapidly switched to the sampling position. In the sampling position, the analyzer was connected directly to the endotracheal tube, isolating the system from the ventilator circuit gas flow and allowing lower airway gas to be withdrawn at a constant flow rate (125 ml/min) during expiration. The flow rate of the analyzer was recorded for each measurement. After one ventilation cycle, the stopcock was returned to the baseline position.

Analyzer signal in nl/l parts per billion (ppb) was relayed to a personal computer running a data acquisition program (LabView; National Instruments, Austin, TX). The baseline signal obtained from ventilator inspiratory circuit gas was analyzed from 15–20 s preceding peak sampling during a single exhalation. Peak signals were defined as consecutive data points varying by <1 ppb that were >2 SD above the mean baseline signal for inspired gas. The minimum duration for a peak signal was set at three times the response time of the analyzer. A representative exhaled NO measure-
ment is shown in Fig. 1. Sampling was not performed if baseline determinations displayed an SD > 0.5 ppb. Peak measurements < 2 SD above mean baseline signal were assigned a value of zero. Measured peak signals ranged as high as 14.7 ppb, and baseline signal in the inspired gas was typically < 0.5 ppb.

At each time point studied, three single breath samples of lower airway gas were obtained at least 30 s apart. To ensure the sampling procedure did not alter ventilation, we compared the PaCO2 before and after triplicate exhaled NO measurements in five animals during the middle of their study course. Pre- and postmeasurement PaCO2 levels were similar, being 49.2 ± 1.1 and 49.8 ± 1.5 mmHg, respectively. Values for exhaled NO were compared among the three samples in a total of 170 sets of triplicate measurements obtained on a total of 21 various study animals in the consortium. For comparison between the first and second measurements, ρ = 0.95; between the second and third measurement, ρ = 0.94; and between the first and third measurement, ρ = 0.97 where ρ refers to correlation coefficient.

VeNOStp was determined based on the ATS recommendations for calculation at constant flow (3, 38). The mean value for peak NO signal from the three exhalation samples at a given time point was employed in the following equation

\[ \text{VeNOStp (nl} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) = 0.896 \times \text{analyser flow rate (l/min)} \times \text{NO (nl/l)} / \text{birth weight (kg)} \]

where 0.896 is the factor to correct to STP.

Statistical analysis. Analysis of variance with Newman-Keuls or Bonferroni post hoc testing was used to compare mean values between more than two groups. Significance was accepted at the 0.05 level of probability. All results are expressed as means ± SE.

RESULTS

NOS enzymatic activity. Changes in NOS enzymatic activity in the proximal lung in the baboon CLD model are shown in Fig. 2. In the control groups, there was a 73% increase in total NOS activity with normal fetal development between 125 and 140 d of gestation (Fig. 2A). In contrast, with 14 d of ventilatory support following preterm birth at 125 d of gestation (CLD), there was an 83% decline in total NOS activity in the proximal lung vs. at birth (125-d-gestation control), and a 90% fall compared with the postconceptional age control (140 d of gestation). Calcium-dependent NOS enzymatic activity, indicative of nNOS or eNOS activity, accounted for the vast majority of total NOS activity, and it rose 65% with normal fetal maturation from 125 to 140 d of gestation (Fig. 2B). However, in lungs from CLD animals, calcium-dependent activity was decreased by 90% compared with that at the time of birth and by 94% compared with an equivalent in utero postconceptional age. The findings for calcium-independent NOS activity are provided in Fig. 2C. Calcium-independent activity was detectable at all ages tested, but it comprised a modest proportion of total NOS enzymatic activity. During normal fetal development from 125 to 140 d of gestation, calcium-independent
activity rose sevenfold. With CLD there was no notable change in calcium-independent NOS activity compared with levels observed at the same postconceptional age in utero.

We determined the amounts of NOS enzymatic activity in the proximal lung derived from nNOS and eNOS by measuring calcium-dependent enzymatic activity in the absence or presence of the nNOS selective antagonist SMTC. Using this approach, we observed that nNOS was the source of the vast majority of NOS activity in all study groups, being 100, 98, and 85% of calcium-dependent activity in 125-d gestation control, CLD, and 140-d gestation control groups, respectively. nNOS-derived activity in lungs from CLD animals was decreased by 92% compared with at the time of birth and by 94% compared with an equivalent in utero postconceptional age (Fig. 3). eNOS-derived activity was not within detectable range in the 125-d gestation control group, and in CLD animals it was <1% of that found in the 140-d gestation control group.

**NOS isoform expression.** The effects of CLD on nNOS expression in the proximal lung are shown in Fig. 4. In the representative immunoblot shown (Fig. 4A), nNOS protein abundance was greater at 140 d compared with 125 d of gestation in the developmental controls. In contrast, nNOS protein was minimally detectable in CLD lung. These observations were confirmed in four independent experiments, which revealed a 2.2-fold increase in nNOS protein levels from 125 to 140 d of gestation during normal fetal maturation, and a 42% decline in nNOS levels with CLD compared with the 125-d-gestation control (Fig. 4B). When evaluated relative to the comparable maturational control (140-d gestation lung), nNOS protein expression was attenuated 73% in CLD lung.

Findings for eNOS expression are displayed in Fig. 5. In the representative immunoblot shown (Fig. 5A), eNOS protein was evident at 125 d of gestation, and it increased dramatically in abundance from 125 to 140 d of gestation. In contrast, in CLD lung eNOS abundance was comparable with that observed at the starting point. Cumulative findings from four separate studies revealed a threefold increase in eNOS protein levels from 125 to 140 d of gestation in the control groups (Fig. 5B). In CLD lung harvested at a postconceptional age of 139 d, eNOS abundance was arrested at the amounts found at 125 d of gestation.

The effects of CLD on iNOS expression in the proximal lung are provided in Fig. 6. In the representative immunoblot shown (Fig. 6A), iNOS protein was minimally detected at 125 d of gestation, and it increased in abundance from 125 to 140 d of gestation. In contrast, in CLD lung obtained at a postconceptional age of 139 d after 14 d of ventilatory support initiated at 125 d of gestation, iNOS abundance was comparable to that observed at the 125-d-gestation starting point. Cumulative findings from four separate studies revealed a 3.3-fold rise in iNOS protein levels from 125 d to 140 d of gestation.
of gestation in the control groups (Fig. 6B). In CLD lung, iNOS abundance remained at the levels observed at 125 d of gestation.

VE\textsubscript{ENO}. In recent studies of exhaled NO levels shortly after birth at 125 d and 140 d of gestation in the baboon, estimates of VE\textsubscript{ENO} were nearly threefold greater in the older age group (34). To provide a longitudinal assessment of airway NO production during the genesis of CLD over the same developmental period, we measured NO output within 1 h of delivery at 125 d of gestation and repeatedly thereafter up until the day before the termination of study at 14 d (Fig. 7). VE\textsubscript{ENO\textsubscript{STP}} remained stable in the range of 0.5–1.0 nl\cdot kg\textsuperscript{-1} min\textsuperscript{-1} from birth to day 7 of life, and it then rose to be 2.5-fold greater than values measured at birth by the end of the second week of life.

**DISCUSSION**

NO plays an important role in the modulation of pulmonary function in the perinatal period (33). In an effort to better understand the contribution of NO to the genesis of CLD associated with preterm birth in humans, in the present study we evaluated the expression and function of the three NOS isoforms in the proximal lung in a baboon model of CLD, which closely mimics the human condition (9, 45). We observed that there is a marked decline in pulmonary NOS expression and activity with CLD, indicative of an attenuated capacity for endogenous NO production in this disease state.

NOS enzymatic activity was evaluated in the proximal lung, where the vast majority of pulmonary nNOS, eNOS, and iNOS in the fetal primate is expressed and localized in respiratory epithelium (34). Enzymatic activity measurements in the presence of excess substrate and cofactors provide a reliable quantitative determination of enzyme abundance, and pharmacological interventions within the activity assay provide the only means to effectively evaluate the relative

![Fig. 5. eNOS expression is decreased in CLD. Immunoblot analysis for eNOS was performed in proximal lung obtained from 125- and 140-d gestation controls (GC, term = 185 d) or following 14 d of ventilatory support after delivery at 125 d of gestation (CLD, A). Signal for eNOS was evident at 135 kDa. Results are representative of 4 independent experiments using samples from different study animals. Summary data for quantitative densitometry for 4 experiments are shown in B. Mean \pm SE values are depicted for protein abundance expressed as percent in 125-d gestation lungs. *P < 0.05 vs. 125 d of gestation; †P < 0.05 vs. 140 d of gestation.](#)

![Fig. 6. Inducible NOS (iNOS) expression is decreased in CLD. Immunoblot analysis for nNOS was performed in proximal lung obtained from 125- and 140-d gestation controls (GC, term = 185 d) or following 14 d of ventilatory support after delivery at 125 d of gestation (CLD, A). Signal for iNOS was evident at 130 kDa. Results are representative of 4 independent experiments using samples from different study animals. Summary data for quantitative densitometry for 4 experiments are shown in B. Mean \pm SE values are depicted for protein abundance expressed as percent in 125-d gestation lungs. *P < 0.05 vs. 125 d of gestation; †P < 0.05 vs. 140 d of gestation.](#)

![Fig. 7. Minute ventilation of NO (VE\textsubscript{ENO}) during the first 2 wk of life in the baboon CLD model. Animals were delivered by hysterotomy, and exhaled NO was measured by chemiluminescence at 1, 8, and 24 h of life and at daily intervals thereafter. NO output is reported as standard temperature and pressure of VE\textsubscript{NO} (VE\textsubscript{ENO\textsubscript{STP}}) in nl\cdot kg\textsuperscript{-1} min\textsuperscript{-1} birth weight. Values are means \pm SE, n = 8, *P < 0.05 vs. days 0–7 of life.](#)
amounts of the three NOS isoforms and their individual contributions to total activity. We found that in contrast to the increase in total NOS enzymatic activity that occurs with normal fetal baboon development during the early third trimester, there was a dramatic decline in NOS activity with the genesis of CLD over the same maturational period. In all study groups, the total NOS activity was primarily calcium dependent, indicative of nNOS and eNOS activity, and the principal change seen with CLD was a fall in calcium-dependent activity. Further studies with selective NOS antagonism revealed that the vast majority of calcium-dependent NOS activity was derived from that isoform. Compared with developmental controls, both nNOS- and eNOS-derived enzymatic activity was decreased by 90% or more in the CLD lung. In contrast to the declines in calcium-dependent NOS isoform activity noted with CLD, the normal severalfold maturational increase in calcium-independent activity was conserved in the CLD lung. These cumulative observations indicate that nNOS is the principal source of NO in the proximal lung during the third trimester in the primate. Furthermore, with CLD, there are dramatic declines in pulmonary nNOS enzymatic activity and also in eNOS enzymatic activity, with the fall in nNOS accounting for the majority of the decline in total activity, whereas that for iNOS is unaltered.

The abundance of NOS isoform proteins was also assessed by immunoblot analysis. Paralleling the observations for nNOS-related enzymatic activity, nNOS protein expression was increased with normal fetal development from 125 to 140 d of gestation, but it was dramatically decreased with the genesis of CLD over the same maturational period. Although the diseases do not have similar pathogeneses, the findings with CLD are comparable with those of patients with obliterative bronchiolitis who display diminished nNOS expression (25). In more general terms, the attenuation of nNOS abundance is similar to that observed in multiple rat tissues following treatment with lipopolysaccharides or interferon-γ (4). In addition, there is evidence from multiple paradigms that nNOS is upregulated by estrogen (14, 43), which normally rises markedly in the fetal circulation during the third trimester due to increasing production by the placenta (29). We postulate that the pulmonary nNOS downregulation observed with CLD may be mediated by a combination of relative estrogen deprivation following premature birth and the inflammatory processes associated with the disorder (20).

eNOS protein abundance was also evaluated by immunoblot analysis. eNOS expression was increased with normal fetal maturation from 125 to 140 d of gestation, but this upregulation was disrupted with preterm birth and the development of CLD. These results in the preterm baboons ventilated from 68 to 76% of gestation mimic the observations made in an ovine model in which immunoblots of both intrapulmonary airways and arteries displayed diminished eNOS protein levels. However, it is important to note that the preterm sheep were ventilated from 84% of gestation to term, so the maturational periods tested in the two models are entirely different, and that the present work is the first to evaluate altered NOS expression with CLD in primates. In addition, the studies in sheep did not evaluate the abundance of nNOS, which we now know is a major source of lung NOS activity in the fetal primate, and the contributions of NOS isoforms to NOS enzymatic activity were not assessed (23).

Similar to the results obtained for nNOS, the decline observed in eNOS expression in CLD is comparable with the findings in patients with obliterative bronchiolitis (25). eNOS is downregulated by inflammatory mediators such as tumor necrosis factor-α (46). Mirroring the above noted hormonal modulation of nNOS, eNOS is also upregulated by estrogen (24). As such, we postulate that fetal estrogen deficiency due to premature birth and/or the inflammatory mechanisms contributing to the genesis of CLD may underlie the diminutions in both nNOS and eNOS in this disorder. Interestingly, in a limited study of postnatal estrogen therapy in preterm infants aimed to improve bone mineralization, CLD was absent in 15 treated infants, whereas it occurred in 4 of 15 controls (41). Further detailed studies will be required in the baboon model to determine the basis of nNOS and eNOS downregulation, whether these alterations can be prevented, and whether doing so has an impact on anatomic and functional pulmonary outcomes.

In contrast to the observed parallel diminutions in nNOS- and eNOS-derived enzymatic activity and nNOS and eNOS protein expression with CLD, there was conservation of the normal maturational increase in calcium-independent NOS activity in the disease model. This was despite observing blunted iNOS protein expression with CLD compared with levels at an equivalent in utero postconceptional age. The basis for the disparity between the changes in iNOS-related enzyme activity, which provides a small fraction of total NOS activity in this paradigm, and iNOS protein levels is unclear. NOS enzymatic activity assays were performed in the presence of excess amounts of substrate and all required NOS cofactors such that enzyme abundance is the limiting factor, and the effective partitioning of calcium-dependent and calcium-independent NOS activity with calcium chelation was confirmed in a cell transfection system expressing each isoform individually. The enzyme activity assays may be more reliably quantified than densitometric analysis of immunoblots because the primary readout for the former is numeric in nature. Thus we conclude that iNOS-derived enzymatic activity is probably conserved during the genesis of CLD in the baboon model, and this would be consistent with the unaltered iNOS protein abundance observed in the preterm lamb model (23). We postulate that, despite the maturational interruption occurring with preterm delivery, iNOS expression is maintained following preterm birth and ventilatory support for 14 d due to upregulation by inflammatory mechanisms related to CLD. The overall contrasting effects of CLD on nNOS and eNOS vs. iNOS mimic the opposing directional changes seen during the genesis of CLD in the baboon model, and this would be consistent with the unaltered iNOS protein abundance observed in the preterm lamb model (23). We postulate that, despite the maturational interruption occurring with preterm delivery, iNOS expression is maintained following preterm birth and ventilatory support for 14 d due to upregulation by inflammatory mechanisms related to CLD. The overall contrasting effects of CLD on nNOS and eNOS vs. iNOS mimic the opposing directional changes seen.
with oblitative bronchiolitis in humans (25) and with lipopolysaccharide and interferon-γ treatment of multiple rat tissues (4).

To provide a longitudinal evaluation of lung NO output during the genesis of CLD, we measured exhaled NO levels as a surrogate, most likely inactive, marker of bioavailable NO. In prior studies we observed almost threefold greater VeNO immediately after birth in baboons delivered at 140 d vs. 125 d of gestation (34). As such, greater NO and NO metabolites may be present to mediate multiple pulmonary functions in the early postnatal period in primates born later compared with earlier in the third trimester. In the present study following premature delivery at 125 d of gestation, VeNO_STP remained stable from birth to day 7 of life, and it then rose to be 2.5-fold greater than values measured at birth by the end of the second postnatal week, suggesting constant and then rising NO and NO metabolite abundance. However, it is important to note that the specific cell types and NOS isoforms responsible for NO gas production have not been determined. In addition, attempts to correlate temporal changes in NO output with the observed alterations in NOS isoform expression and activity with CLD do not take into account possible differences in endogenous NOS inhibition by asymmetric dimethyl arginine or in arginine metabolism by arginases and other mechanisms (22, 44). Despite these potential limitations, the present work provides important new information about alterations in NOS isoform abundance and NO production in a primate model performed at a developmental stage equivalent to 27-wk postconceptional age in humans that closely mimics the current form of CLD following premature birth in humans (9, 45). Studies of the impact of NOS isoform-selective antagonists on exhaled NO should now be considered in the preterm baboon model.

The changes in VeNO_STD demonstrated in the current work in primates complement the information related to NO and NO metabolites from human studies. Besides generating S-nitroscysteines and S-nitrosothiols, NO can react with O2 and reactive oxygen species to yield higher oxides of nitrogen and peroxynitrite. Peroxynitrite reacts with proteins to form 3-nitrotyrosine (17). Banks and colleagues (5) measured plasma 3-nitrotyrosine levels following birth in premature infants with CLD and demonstrated that levels are greater than in preterms without CLD. In addition, in the CLD group the levels rose during the latter half of the first month, generally paralleling the increase in VeNO_STD that we report here. Vyas and coworkers (42) reported stable nitrate levels in bronchoalveolar lavage (BAL) early in life, which then rose in the second week in infants with CLD, also paralleling the present findings for NO output in the preterm CLD model. In contrast, BAL nitrate levels fell dramatically at 14 days of life in premature infants without CLD. The authors do not comment on how their subjects tolerated BAL. In the premature baboon, the BAL procedure results in significant hemodynamic and pulmonary instability. As such, BAL samples are not feasible in the baboon model before necropsy. It should be noted that Storme et al. (39) evaluated exhaled NO levels in infants with CLD and demonstrated greater levels than in controls, but the studies were done from 1 mo of postnatal age onward. At any rate, the present findings for lung NO output in the baboon model of CLD are generally consistent with related available information in premature infants studied over a comparable developmental period.

The functional implications of decreased pulmonary nNOS and eNOS expression and diminished NO production by those isoforms in the baboon CLD model are multiple. Studies in newborn piglets, using both intact animals and isolated tracheae, indicate that epithelium-derived NO counteracts bronchoconstriction (19, 27). There is also evidence that epithelium-derived NO modifies distal lung function; studies in fetal lambs indicate that NO regulates lung liquid production (10), and further work in the newborn piglet reveals a role for NO in modulating lung compliance (27). Furthermore, the critical contribution of NO to pulmonary vasomotor regulation in the perinatal period is well established (33), and investigations in fetal lambs indicate that nNOS is an important source of endogenous NO impacting on the lung circulation (28). It is possible that proximally expressed nNOS modulates pulmonary vascular and distal airway and parenchymal function via hemocrine pathways and airway lining fluid communications. With these multiple considerations in mind, we postulate that diminished nNOS- and eNOS-derived NO and NO metabolites may contribute not only to the airway dysfunction but also to the parenchymal and pulmonary vascular abnormalities that are characteristic of CLD. Studies of the impact of NO replacement in the baboon CLD model are now warranted to clarify the contribution of relative NOS deficiency to the morphological and functional consequences of the disorder.

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