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 NO isoforms, neuronal NO (nNOS), endothelial NO (eNOS), and inducible NO (iNOS), are primarily expressed in proximal respiratory epithelium in the developing primate. In addition, there are maturational increases in the expression of the three NO 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 primates 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 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 due to 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; Vitaltrends 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 goals, FiO2 was weaned until <0.40, and then modifiers of mean airway pressure or FiO2 were decreased as tolerated. 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). Experiments were performed on the most 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

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 due to tissue availability.
zymatic activity assays in the absence and presence of the
dence of NOS activity was evaluated by the addition of 2.5
[3H]L-citrulline was collected in scintillation vials and quan-
(Tris form) and eluted with 1 ml of the 40 mM HEPES buffer.

pH 5.5, with 2 mM EDTA and 2 mM EGTA. The terminated
arginine. After incubation at 37 °

l, Doraville, GA) zero gas
ders were washed in the 150 mM NaCl
fi

Times obtained from nonintubated adult volunteers have
les obtained from a distal endotracheal port. Additionally, single breath
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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 interfer-
ence 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 endotrache-
tube. In the baseline position the animal was continu-
ously 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 ven-
tilation cycle, the stopcock was returned to the baseline
position.

Analyzer signal in n/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 for 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 dura-
tion 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, r = 0.95; between the second and third measurement, r = 0.94; and between the first and third measurement, r = 0.97 where r refers to correlation coefficient.

\( \text{VeNO}_{\text{STP}} \) 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

\[
\text{VeNO}_{\text{STP}} (\text{nl} \cdot \text{kg}^{-1} \cdot \text{min}^{-1}) = 0.896 \times \text{analyzer flow rate (l/min)} \times \text{NO (nl/l)} \div \text{birth weight (kg)}
\]

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.

V˙ENO. In recent studies of exhaled NO levels shortly after birth at 125 d and 140 d of gestation in the baboon, estimates of V˙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). V˙ENOstP remained stable in the range of 0.5–1.0 nl·kg⁻¹·min⁻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
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 nNOS 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. Parallelizing 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 eNOS and accounting for the majority of the decline in total activity, whereas that for iNOS is unaltered.

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. 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 NOS and iNOS vs. iNOS mimic the opposing directional changes seen.
with obliterative 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, VeNOSTP 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 VeNOSTP demonstrated in the current work in primates complement the information related to NO and NO metabolites from human studies. Besides generating 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 VeNOSTP 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 primate 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 trachea, 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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