Role of endogenous nitric oxide in hyperoxia-induced airway hyperreactivity in maturing rats

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Received 10 September 1999; accepted in final form 6 December 1999

Iben, Sabine C., Ismail A. Dreshaj, Carol F. Farver, Musa A. Haxhiu, and Richard J. Martin. Role of endogenous nitric oxide in hyperoxia-induced airway hyperreactivity in maturing rats. J Appl Physiol 89: 1205–1212, 2000.—We sought to define the effects of maturation and hyperoxic stress on nitric oxide (NO)-induced modulation of bronchopulmonary responses to stimulation of vagal preganglionic nerve fibers. Experiments were performed on decerebrate, paralyzed, and ventilated rat pups at 6–7 days (n = 21) and 13–15 days of age (n = 23) breathing room air and on rat pups 13–15 days of age (n = 19) after exposure to hyperoxia (≥95% inspired O2 fraction for 4–6 days). Total lung resistance (RL) and lung elastance (EL) were measured by body plethysmograph. Vagal stimulation and release of acetylcholine caused a frequency-dependent increase in RL and EL in all animals. The RL response was significantly potentiated in normoxic animals by prior blockade of nitric oxide synthase (NOS) (P < 0.05). Hyperoxic exposure increased responses of RL to vagal stimulation (P < 0.05); however, after hyperoxic exposure, the potentiation of contractile responses by NOS blockade was abolished. The response of EL was potentiated by NOS blockade in the 13- to 15-day-old animals after both normoxic and hyperoxic exposure (P < 0.01). Morphometry revealed no effect of hyperoxic exposure on airway smooth muscle thickness. We conclude that NO released by stimulation of vagal preganglionic fibers modulates bronchopulmonary contractile responses to endogenously released acetylcholine in rat pups. Loss of this modulatory effect of NO could contribute to airway hyperreactivity after prolonged hyperoxic exposure, as may occur in bronchopulmonary dysplasia.

Release of endogenous nitric oxide (NO) has been implicated in airway smooth muscle relaxation. Specifically, this would serve as a mechanism to attenuate contractile responses elicited by cholinergic or other excitatory substances (14). Inhibition of NO by nonspecific nitric oxide synthase (NOS) inhibitors has been shown under in vitro conditions to enhance tracheal contractile responses to electrical field stimulation (EFS) in adult guinea pigs (4) and humans (29). Comparable studies have not been performed under in vivo conditions. Potter et al. (22) showed that Nω-nitro-L-arginine methyl ester (L-NAME) induces an increase in baseline total lung resistance (RL) in 2- to 5-day-old piglets that is reversed by inhaled NO. One purpose of our study was therefore to delineate the effect of NOS blockade on airway contractile responses elicited by endogenously released acetylcholine after stimulation of vagal preganglionic nerve fibers in rat pups under in vivo conditions.

The balance between contractile and relaxant responses of airway smooth muscle is altered in hyperreactive airways. In preterm human infants, hyperoxic exposure is widely believed to contribute to the development of bronchopulmonary dysplasia (BPD). Furthermore, increased airway resistance has been observed in this disorder, and infants with BPD are predisposed to subsequent airway hyperreactivity (2, 21). Tracheal smooth muscle from rat pups and piglets exposed to prolonged hyperoxia exhibits increased contractile responses to endogenously released acetylcholine but not to inhaled acetylcholine (1, 14). Inhaled acetylcholine and systemic acetylcholine also induce greater increases in RL in 4-wk-old rat pups after exposure to hyperoxia compared with normoxic controls (12). The mechanism by which prolonged hyperoxic exposure induces such an increase in airway reactivity is not fully understood. The possibility exists that hyperoxic exposure causes impairment in the ability of endogenously released relaxing factors, including NO, to induce airway relaxation, which in turn might potentiate airway contractile responses.

In this study, we developed an animal model for characterizing changes in RL and lung elastance (EL) induced by vagal stimulation during early postnatal life. This allowed us to test the hypotheses that 1) activation of vagal preganglionic nerve fibers induces NO release, 2) NO is an important modulator of bronchopulmonary contractile responses at this age, and 3) this modulation of RL by NO is impaired after hyperoxic exposure.

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METHODS

Animal model. Newborn Sprague-Dawley rat pups were obtained with their mothers. Animals from multiple litters were studied at 6–7 days of age (21 animals) and at 13–15 days of age (23 animals). All of these animals were maintained in room air before the study. An additional 19 animals from multiple litters were exposed to ≥95% oxygen for 4–6 days before study at 13–15 days of life. The animals were housed with their mothers in an exposure chamber (38 liter) with a continuous flow of oxygen of 11 l/min to maintain inspired \( O_2 \geq 95\% \) or in a commercial rat cage in room air. Water and food were provided ad libitum. Nursing mothers were rotated between hyperoxic- and room air-exposed litters every 24 h to avoid oxygen toxicity in the mothers and to eliminate maternal effects between groups.

Experimental protocol. To avoid the confounding effect of anesthesia, decerebration was performed before the experimental protocol was initiated. Under Metofane anesthesia, the animals were decerebrated in the following manner. A midline skin incision was made over the interparietal bone, and the skull was penetrated with sharp forceps approximately midway between the lambdoid and the occipital suture. A looped wire was inserted through the bone defect and gently swung bilaterally. Transsection at the midcollicular level was confirmed by autopsy after the end of the study in randomly selected animals. The animals were then tracheotomized just below the larynx, and a tracheal cannula consisting of PE-90 tubing (6- to 7-day-old animals, 1.27 mm OD) or PE-160 tubing (13- to 15-day-old animals, 1.57 mm OD) was cut to 1.5 cm, inserted, and connected to a Harvard rodent ventilator (model 683, adjusted for small tidal volumes) and by a side port to a pressure transducer. The animals were ventilated with 100% inspired \( O_2 \) fraction, a tidal volume of 7 µl/g, and a rate of 90/min. A venous catheter (PE-10 tubing, 0.61 mm OD) was introduced in the external jugular vein for venous access. The animal was then placed in a head-out body plethysmograph made from a 120-ml syringe. Appropriate sealing was achieved with a latex collar fastened to the proximal opening of the plethysmograph. The animal was paralyzed with gallamine (10 µg/g), and doses were repeated as needed to suppress spontaneous respiratory efforts and movements. Propranolol (1 µg/g) was given intravenously for β-sympathetic blockade before vagal stimulation.

Vagal stimulation and NOS blockade. Bilateral high cervical vagotomy was performed in all animals, and the distal ends of both vagi were placed on bipolar stimulating electrodes. For each age group, studies were performed in two sets of animals, only one of which received L-NAME for NOS blockade as follows: 12 of 21 animals studied at 6–7 days of age, 12 of 23 animals studied at 13–15 days of age under normoxic conditions, and 10 of 19 animals studied after hyperoxic exposure. L-NAME (30 µg/g) was administered intravenously 20 min before vagal stimulation. After baseline values of Rt and El were obtained, bilateral vagal stimulation was applied for 5 s with a constant current (0.5 mA) and increasing frequencies (1–32 Hz) at 3-min intervals. A 20-s recording was obtained, and the five respiratory cycles at peak response after vagal stimulation were analyzed and averaged. Resistance and elastance were calculated by ANADAT software using the least-squares technique. At the end of the experiment, stimulation with 16 Hz was repeated to ensure reproducibility of the response. Four animals received intravenous atropine (1 µg/g), and the response to a 16-Hz stimulation was again recorded.

Lung histology and airway morphometric analysis. An additional seven normoxic and seven hyperoxic rat pups were killed with an overdose of pentobarbital, and their lungs were inflated with 10% formalin at a pressure of 20 cmH\(_2\)O for 15 min and stored in formalin. Fixed lungs were cut into 5-mm sagittal sections and embedded in paraffin; 5-µm sections were then placed on charged microscopic slides. Sections were stained with hematoxylin and eosin. A pathologist (Farver) blinded to the treatment group analyzed the sections for qualitative histological changes. Unstained microscopic slides of paraffin sections were rehydrated and incubated with a smooth muscle actin antibody (Dako) by the avidin-biotin peroxidase complex technique and counterstained with hematoxylin. Phosphate-buffered saline was substituted for the primary antibody as a negative control. The smooth muscle area of airways ≤ 1,500 µm diameter was quantified using a BIOQUANT True Color Windows 95, version 2.0 system (R&M Biometrics, Nashville, TN). The measured smooth muscle area for each airway was divided by the internal circumference (length of the epithelial basement membrane) of the airway to calculate the smooth muscle thickness for each airway. To exclude airways with oblique cross sections, a longest (L) and short (S) internal diameter were measured and only those airways with an S-to-L ratio of ≥0.5 were used for quantification.

Statistical analysis. The effect of L-NAME under normoxic conditions at two ages, the effect of hyperoxia vs. normoxia at 13–15 days, and the effect of L-NAME after normoxic and hyperoxic exposure at 13–15 days were all compared by two-way ANOVA. Baseline Rt and El values before and after L-NAME were compared by paired \( t \)-test. Baseline Rt and El between normoxia- and hyperoxia-exposed animals were compared by unpaired \( t \)-test. The mean smooth muscle thickness of normoxia- and hyperoxia-exposed animals was compared by unpaired \( t \)-test. \( P \) values <0.05 were considered significant.

RESULTS

Effects of postnatal maturation on lung mechanics and response to vagal preganglionic nerve fiber stimulation. Postnatal maturation from 6–7 days to 2 wk was associated with a decrease in Rt (4.35 ± 0.19 vs. 1.79 ± 0.05 cmH\(_2\)O·ml\(^{-1}·s\); \( P < 0.001 \)) and a decline in El (81.14 ± 2.4 vs. 42.74 ± 2.2 cmH\(_2\)O/ml; \( P < 0.001 \)). Bilateral stimulation of the cervical vagus nerves produced rapid increases in Rt and El. Representative data for tracheal pressure and flow responses to vagal stimulation are presented in Fig. 1. The relative magnitude of the response of Rt and El, when expressed as a change from baseline, appeared to decrease with advancing age (Fig. 2).

Effects of NOS blockade on the Rt response to vagal stimulation. We initially evaluated the effect of L-NAME on baseline Rt and El as well as on the response to vagal stimulation under normoxic conditions at the two postnatal ages. In the 6- to 7-day-old rat pups, baseline Rt did not change significantly at treatment with L-NAME (4.16 ± 0.22 vs. 4.36 ± 0.13 cmH\(_2\)O·ml\(^{-1}·s\); \( P = 0.22 \)). In the 13- to 15-day-old animals, Rt was significantly higher after NOS blockade (1.69 ± 0.05 vs. 1.90 ± 0.07 cmH\(_2\)O·ml\(^{-1}·s\); \( P < 0.05 \)). We compared change in Rt over baseline before and after L-NAME (rather than absolute resistance) to avoid the confounding effect of the change in baseline Rt after L-NAME observed at 13–15 days. L-NAME-
treated animals exhibited a greater response of Rt. to vagal stimulation compared with controls at both ages (6–7 days, \( P < 0.02 \), Fig. 3, left; and 13–15 days, \( P < 0.01 \), Fig. 3, right).

The effect of vagal stimulation on Rt. was subsequently compared between normoxic- and hyperoxic-exposed 13- to 15-day-old animals. Baseline values of Rt. were not significantly different between normoxic and hyperoxic rat pups (\( P > 0.1 \)). Furthermore, NOS blockade did not change baseline Rt. in the hyperoxic animals (1.69 ± 0.07 vs. 1.78 ± 0.12 cmH\(_2\)O·ml\(^{-1}\)·s, \( P = 0.1 \), before and after L-NAME). Contractile responses to increasing frequencies of vagal stimulation were significantly enhanced in animals after hyperoxic exposure (\( P < 0.05 \), Fig. 4). In contrast to normoxic animals, NOS blockade with L-NAME in the 13- to 15-day-old hyperoxia-exposed animals did not result in further enhancement of Rt. responses (\( P > 0.7 \), Fig. 4). Treatment with intravenous atropine abolished all responses to vagal stimulation.

**Effects of NOS blockade on the EL response to vagal stimulation.** Treatment with L-NAME resulted in a significant increase in baseline EL at both 6–7 days (79.25 ± 3.53 vs. 93.54 ± 3.29 cmH\(_2\)O/ml, \( P < 0.02 \)) and 13–15 days of age (44.05 ± 3.37 vs. 49.83 ± 3.86 cmH\(_2\)O/ml, \( P < 0.01 \)). There was an increase in EL in response to vagal stimulation in all groups (\( P < 0.01 \)). There was no effect of L-NAME on the response of EL to vagal stimulation in 6- to 7-day-old animals (\( P > 0.05 \)). However, NOS blockade resulted in significantly enhanced responses of elastance in the 13- to 15-day-old animals, after both normoxic (\( P < 0.01 \), Fig. 5, left) and hyperoxic exposure (\( P < 0.01 \), Fig. 5, right). As shown in Fig. 5, exposure to hyperoxia resulted in enhanced

Fig. 1. An example of the responses of tracheal pressure and air flow to increasing frequencies of vagal stimulation (1–32 Hz) in a 14-day-old decerebrate, paralyzed, and mechanically ventilated rat. Brackets represent duration of vagal stimulation.

Fig. 2. Effect of increasing frequencies of bilateral vagal stimulation on changes in lung resistance (Rt, left) and lung elastance (EL, right) at 6–7 days (●) and 13–15 days (○) of age. Each data point represents mean ± SE. Responses of Rt. and EL at the younger age were of greater magnitude.
responses of elastance to vagal stimulation compared with normoxic controls ($P < 0.03$).

**Histological examination.** There were no histological differences by light microscopy between the lungs from hyperoxic and those from normoxic animals. The lungs from both groups showed mildly hyperplastic bronchus-associated lymphoid tissue, a nonspecific finding commonly seen in laboratory rodents. There was no evidence of bronchiolar epithelial or alveolar injury. Morphometric studies revealed no significant difference in airway smooth muscle thickness between hyperoxic and normoxic animals ($2.22 \pm 0.73$ vs. $2.56 \pm 0.63 \mu m$, for normoxic vs. hyperoxic animals, respectively; Fig. 6).

**DISCUSSION**

NO has been implicated as an important modulator of airway smooth muscle contractility in adult animals (4, 8, 17) and humans (29). However, during early postnatal maturation, there is little information on the role of endogenously released NO in attenuating airway contractile responses, despite the relatively small caliber and high resistance of immature airways. Such a high baseline RL during early maturation was confirmed in the current study and is in agreement with our earlier studies in maturing piglets (9). Furthermore, the greater responses of both RL and Et that we observed in response to vagal stimulation are probably related to the small size of immature airways from 1-wk-old as opposed to 2-wk-old rat pups under baseline conditions.

We have shown for the first time under in vivo conditions that airway responsiveness to cholinergic stimulation is attenuated by endogenously released NO during early maturation. There is an abundance of information about the relaxant effect of NO on tracheal responsiveness under in vitro conditions in adult animals of different species as well as in humans. The response of adult guinea pig trachea to EFS but not to exogenous acetylcholine is potentiated by prior NOS blockade (4). Enhancement of contractile responses to EFS after l-NAME has also been shown in human trachea as well as segmental and subsegmental bronchi (29). Our group has previously shown in piglets that tracheal contractile responses to exogenous ace-
tylcholine are enhanced after NOS blockade at less than 1 wk and 2–3 wk of age but not in 3-mo-old animals (14). Consistent with these findings, precontracted trachea from rat pups exhibits a relaxant response to substance P, which decreases with advancing postnatal age and is diminished after incubation with L-NAME (18). Therefore, under in vitro conditions, NO appears to be an important modulator of tracheal constrictor responses, especially during early development.

Available information on the relaxant mechanisms modulating airway contractility under in vivo conditions has been derived from adult animals. Adult guinea pigs (17) and rabbits (7) exhibit increased airway contractile responses to histamine after NOS blockade, which suggests that NO is an important modulator of airway contractile responses in different species. This mechanism seems to be impaired in states of induced airway hyperreactivity (7, 17). Comparable in vivo studies have not been done in young animals. Waldron et al. (28) demonstrated in 2- to 11-day-old kittens that vagal stimulation causes airway relaxation after blockade of cholinergic and adrenergic pathways, suggesting the presence of a functional

Fig. 5. Effect of bilateral vagal stimulation on change in $\Delta EL$ in 13- to 15-day-old normoxic and hyperoxic rat pups after treatment with L-NAME (○) compared with controls (●). Each data point represents mean ± SE. Left: 13- to 15-day-old animals reared in room air. Right: 13- to 15-day-old animals after a 4- to 6-day exposure to hyperoxia. Addition of L-NAME resulted in enhanced responses of $\Delta EL$ to vagal stimulation.

Fig. 6. Photomicrograph of immunostained smooth muscle cells in cross section through a bronchiole in a hyperoxic rat lung. B, bronchiole; arrow, smooth muscle. Original magnification = ×400. There was no morphometric difference between normoxia- and hyperoxia-exposed rat pups.
nonadrenergic inhibitory innervation early in life. In contrast, Colasurdo et al. (7) found that the inhibitory nonadrenergic-noncholinergic (iNANC) response to EFS was absent in 1-wk-old rabbits, whereas older animals exhibited tracheal relaxation. These studies demonstrate species differences in the presence and maturation of relaxant iNANC responses in the newborn period. Nonetheless, Colasurdo et al. (7) observed that the relaxant iNANC response to EFS was significantly decreased in ragweed-sensitized rabbits at 2, 4, and 12 wk of age, which suggests dysfunction of iNANC innervation under these conditions.

We have demonstrated in the 13- to 15-day-old rat pups that there is significantly increased airway reactivity after prolonged exposure to hyperoxia. Szarek et al. (26) showed in adult rats that there is an increase in sensitivity of primary bronchi to EFS after 3 days of hyperoxic exposure that could not be attributed solely to an increase in smooth muscle area. Comparable findings were made in newborn guinea pigs (26) and rat pups (13) under in vitro conditions. This has been replicated under in vivo conditions by Hershenson et al. (12) who studied rat pups after 1 wk of hyperoxic exposure beginning at 21 days. Airway responsiveness to both intravenous and inhaled acetylcholine was increased in hyperoxia-exposed animals compared with controls. In contrast to our study, Hershenson et al. (12) reported an increase in airway smooth muscle thickness after hyperoxic exposure. These morphometric differences from our own data might be attributable to the fact that we studied younger animals and employed a different technique, using measurement of the circumferential muscle area rather than airway thickness. Our data suggest that loss of relaxation after hyperoxic injury cannot be primarily attributed to morphological changes.

NOS is present in airway epithelium, nerve endings, and possibly airway smooth muscle cells (25), and NO is an important neurotransmitter of the iNANC pathway. NOS expression is known to be developmentally regulated (20). In the absence of specific NOS blockade, our study does not allow us to differentiate which NOS isoform is preferentially involved in modulating airway contractility. All isoforms of NOS are expressed in airway epithelium (20, 25), and nerve fibers of the iNANC system containing neural NOS (nNOS) and innervating airway smooth muscle and epithelium of humans (10) are present early in postnatal life and subsequently decrease with age (6). Our group has previously demonstrated an increase in endothelial NOS and inducible NOS (iNOS) in pulmonary tissue of 21-day-old rats after exposure to hyperoxia for 1 wk, compared with normoxic controls (23). In that study, we evaluated NOS expression in the whole lung, so it is unclear whether the elevation of NOS isoforms occurred in airways or vascular or other pulmonary tissues (23). Nonetheless, it appears that the failure of NOS blockade to further increase airway contractility after hyperoxic exposure is not due to depletion of NOS but rather to functional impairment of downstream signaling mechanisms mediating the physiological effects of NO on airway function.

Our study does not specifically address the site of airway constriction that is modulated by NO release. However, intratracheal intubation did bypass part of the trachea in our model. In rats, airways, including the most distal lung units, are innervated by airway-related preganglionic neurons (11). Furthermore, immunohistochemical studies of the lamb lung at different ages (fetal, newborn, and adult) demonstrate that all isoforms of NOS are expressed in bronchial and proximal bronchiolar epithelium, whereas iNOS can also be found in terminal and respiratory bronchioles and nNOS in epithelium at all levels including the alveolar wall (25). Therefore, interplay between cholinergic innervation and NO expression in proximal and distal airways as well as parenchymal contractile elements could be contributing to the phenomena that we have observed. A role for endogenous NO in regulating peripheral contractile elements is supported by our prior observation that NOS blockade increases more tissue than airway resistance in open-chested piglets (22).

Evaluation of EL as well as RL responses to vagal stimulation allows us to attempt to differentiate the effect of NO on both central and peripheral contractile elements. It is known that narrowing of the airway tree induced by bronchoconstrictive stimuli is also associated with an increase in lung stiffness (19), which cannot be explained by bronchoconstriction alone (3) and could be due to contraction of smooth muscle in the periphery (3). Elevation of EL in response to vagal stimulation may be explained by contraction of alveolar interstitial contractile elements or periductal smooth muscle. This is supported by our recent neuroanatomic studies in rats showing that most distal airways are innervated by vagal preganglionic neurons (11) and are under cholinergic control (16).

In our study, we have shown that the increase in EL may occur independently of the increase in RL. NOS blockade in the 6- to 7-day-old day animals had no effect on changes in EL in response to vagal stimulation, despite the fact that the response of RL was potentiated. This suggests that, at that age, relaxant effects of NO in peripheral lung units are not as prominent as in the more central airways. In contrast, in 13- to 15-day-old animals, there was significant potentiation of EL responses after NOS blockade, which was preserved after hyperoxic exposure, whereas there was no comparable effect of NOS blockade on RL after hyperoxic exposure. This leads us to speculate that, in the 2-wk-old rat pups, NO modulates both central and peripheral contractile elements, whereas hyperoxic injury primarily affects the ability of NO to modulate airway contractile responses in the central airways.

Potential limitations of the present study are that intravenous L-NAME might have widespread systemic effects such as pulmonary and systemic hypertension. It was not feasible to measure blood pressure in our model; however, it is unlikely that changes in arterial blood pressure would explain our findings. It is possible...
that NOS blockade may have decreased bronchial blood flow and altered bronchoconstrictor responses; however, in sheep, alteration in bronchial vascular tone was not associated with changes in baseline RI or bronchoconstrictor responses (24). Because the effects of L-NAME may be only partially reversible, we could not randomize the order of measurements before and after L-NAME administration. To avoid this time effect, we therefore chose to use different treatment groups (L-NAME vs. no L-NAME) within litters. Vagal stimulation as performed in this study may have activated extrinsic sympathetic or iNANC nerve fibers containing other neurotransmitters like vasoactive intestinal peptide and neuropeptide Y (5); however, β-adrenergic responses were blocked by intravenous propranolol. It is possible that vagal stimulation may have caused release of substance P from afferent nerve endings and contributed to NO release, although SP also enhances cholinergically mediated contractile responses (1, 18). The fact that relaxant responses to stimulation of vagal preganglionic nerve fibers are abolished after ganglionic blockade (31) suggests that the observed changes in response to stimulation of preganglionic nerve fibers following NOS blockade are under vagal efferent control, which is in disagreement with studies by Watson et al. (30).

In summary, the results of this study have shown that endogenously released NO is important for the regulation of airway smooth muscle activity in early postnatal life, modulating airway and parenchymal responses to endogenously released acetylcholine. In response to hyperoxic exposure, NO appears to be impaired in its ability to modulate constriction of central airways but not constriction of the most distal bronchopulmonary units. We speculate that this has important implications for the pathogenesis of BPD and the subsequent development of airway hyperreactivity in former preterm infants.

This work was supported by National Heart, Lung, and Blood Institute Grants HL-58470 and HL-50527 and the Ross Products Division of Abbott Laboratories. S. C. Iken is supported by the William Randolph Hearst Neonatology Fellowship Endowment at Rainbow Babies and Children’s Hospital.

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