Disruption of NO-cGMP signaling by neonatal hyperoxia impairs relaxation of lung parenchyma

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Sopi RB, Haxhiu MA, Martin RJ, Dreshaj IA, Kamath S, Zaidi SI. Disruption of NO-cGMP signaling by neonatal hyperoxia impairs relaxation of lung parenchyma. Am J Physiol Lung Cell Mol Physiol 293: L1029–L1036, 2007. First published July 27, 2007; doi:10.1152/ajplung.00182.2007.—Exposure of immature lungs to hyperoxia for prolonged periods contributes to neonatal lung injury and airway hyperreactivity. We studied the role of disrupted nitric oxide-guanosine 3’,5’-cyclic monophosphate (NO-cGMP) signaling in impairing the relaxant responses of lung tissue from hyperoxia-exposed rat pups. Pups were exposed to ≥95% O2 or room air for 7 days starting from days 1, 5, or 14. The animals were killed, lungs were removed, and 1-mm-thick lung parenchymal strips were prepared. Lung parenchymal strips of room air or hyperoxic pups were preconstricted using bethanechalor and then graded electrical field stimulation (EFS) was applied to induce relaxation. EPS-induced relaxation of lung parenchymal strips was greater at 7 and 12 days than at 21 days in room air-exposed rat pups. Hyperoxic exposure significantly reduced relaxation at 7 and 12 days but not 21 days compared with room air exposure. NO synthase blockade with Nω-nitro-L-arginine methyl ester diminished relaxant responses in room air but not in hyperoxic pups at 12 days. After incubation with supplemental l-arginine, the relaxation response of hyperoxic strips was restored. cGMP, a key mediator of the NO signaling pathway, also decreased in strips from hyperoxic vs. room air pups and cGMP levels were restored after incubation with supplemental l-arginine. In addition, arginase activity was significantly increased in hyperoxic lung parenchymal strips compared with room air lung parenchymal strips. These data demonstrate disruption of NO-cGMP signaling in neonatal rat pups exposed to hyperoxia and show that bioavailability of the substrate L-arginine is implicated in the predisposition of this model to airway hyperreactivity.

Respiratory morbidity in the form of bronchopulmonary dysplasia (BPD) is a substantial clinical problem in the survivors of neonatal respiratory disease (21). Increased airway reactivity in childhood is the most common longer term manifestation of neonatal lung injury and is most pronounced when there is a history of BPD (15, 35). Therefore, animal models have been developed to simulate neonatal lung injury in which immature animals are exposed to various combinations of assisted ventilation and supplemental oxygen (7, 28). We and others demonstrated in rat pups that exposure to high inspired oxygen is associated with many pathophysiological features of BPD, including increased constrictor responses of tracheal tissue and greater increases in lung resistance during cholinergic stimulation under in vitro and in vivo conditions, compared with room air-exposed controls (4, 17, 18, 24, 30, 39).

Excessive airway smooth muscle contraction may be a consequence of impaired relaxation in contractile elements situated in large airways or lung parenchyma (18, 30). Our previous studies demonstrated that endogenous nitric oxide (NO) opposes the excitatory cholinergic contribution to lung resistance of both airway and tissue contractile elements in open-chested ventilated piglets (22). Furthermore, under in vivo conditions, the potentiation of lung resistance responses to vagal stimulation after blockade of NO synthase (NOS) in normoxic rat pups was abolished in hyperoxia-exposed animals (17). These findings point to a modulatory effect of NO on extrapulmonary or intrapulmonary airway contractile elements that appear to be lost after hyperoxic exposure, as might occur in neonatal lung injury. As NO is produced from L-arginine via NOS (32), bioavailability of L-arginine may play a key role in the production of NO and thus its effects on lung function.

We sought to test the hypothesis that relaxant mechanisms in lung parenchymal structures are enhanced in early postnatal life and, therefore, most vulnerable to hyperoxic exposure at this age via disruption/alteration of NO-mediated signaling pathways. In this study, we employed lung parenchymal strips from neonatal rat pups as our prior studies suggest a role for NO in modulating peripheral bronchopulmonary contractile elements. Use of parenchymal tissue allows us to directly compare physiological measurements with changes in cGMP production and assess a role of L-arginine bioavailability under conditions of neonatal hyperoxic exposure.

Materials and Methods

Animal and lung parenchymal strip preparation. Experiments were performed in rat pups (Sprague-Dawley) of different ages. The animal protocol was approved by Institutional Animal Care and Use Committee of Case Western Reserve University, Cleveland, OH. On day 1, 5, or 14 of life rat pups were exposed to hyperoxic (≥95% O2) or room air conditions for a period of 7 days. Rat pups from two different litters were randomly mixed at each age group and assigned to hyperoxic (n = 10) or room air (n = 10) groups at each age. Hyperoxic groups were housed with their mothers in a Plexiglas chamber (38 l) and exposed to continuous flow of O2 (2 l/min) for 7 days. Mothers were rotated each day between room air and hyperoxic groups to protect them from a constant hyperoxic exposure. Oxygen concentration was monitored continuously via oxygen analyzer (MiniOX-I, MSA Medical Products, Pittsburgh, PA). The pups as-
signed to room air were kept in a commercial rat cage in room air. Animals were euthanized by asphyxiation in CO2 on days 7, 12, or 21. It was not possible to process all lung parenchymal strips on the same day; therefore, some animals were killed after being returned to 21% O2 for 24 h, such that exposure to hyperoxia was always of 7 days duration. No differences were observed in the relaxation of lung parenchymal strips of animals that were killed either immediately or 1 day after cessation of O2 exposure.

Lungs were removed and placed in ice-cold oxygenated Krebs-Henseleit (KH) solution (concentration in mM: 118.2 NaCl, 25 NaHCO3, 4.6 KCl, 1.2 KH2PO4, 1.2 MgSO4, 2.5 CaCl2, and 10% dextrose, pH 7.4). Lung parenchymal strip of 1-mm thickness was obtained by sectioning of the left lung of each animal in an apex-base axis using a vibratome (Vibratome-1000 Plus Sectioning System). The lung parenchymal strips were transferred to 20-ml tissue baths (Radnotti Glass, Glass Technology, Monrovia, CA) containing KH solution and continuously oxygenated with a gas mixture (95% O2-5% CO2) at 37°C.

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Physiological studies. One end of each lung parenchymal strip was anchored at the bottom of the tissue bath and the other end was connected to a force displacement transducer (TF 03C, Grass Instruments, Quincy, MA) as we described previously for tracheal cylinders (2). An initial load of 0.2 g was used. The suspended lung parenchymal strips were rinsed every 20 min with KH solution and allowed to equilibrate for 60 min to establish a steady baseline. Lung parenchymal strip tension was recorded by Power Lab/4SP AD Instruments using Chart 5.0 Software. A cumulative concentration-response curve was made to find a concentration of bethanechol (Sigma) that elicited 50–75% of maximal response in lung parenchymal strips. A concentration of bethanechol of 10⁻⁴ M was found to be optimal to elicit 50–75% of a maximal response. Electrical field stimulation (EFS) was applied through platinum wire electrodes to preconstricted lung parenchymal strips at various voltages (5–50 V AC at 60 Hz) for 10 s at 2-min intervals to induce relaxation. The relaxation of strips after EFS was expressed as a percentage of total preconstricted state for each strip was previously described by us (30) and compared between ages for room air and hyperoxic groups.

Because comparable relaxation responses were observed in pups studied at 7 and 12 days, we arbitrarily used 12-day-old pups for all further studies related to understanding the mechanisms by which hyperoxia impairs lung parenchymal relaxation. We sought to determine whether relaxant mechanisms are disrupted during hyperoxia via alteration in NO-mediated signaling. Lung parenchymal strips from room air and hyperoxic groups (each n = 10) were incubated with a NOS inhibitor, Nω-nitro-L-arginine methyl ester (L-NAME; 10⁻⁴ M, Sigma), for 30 min, before preconditioning with bethanechol and then exposed to EFS. Responses obtained without the presence of L-NAME served as controls.

To study the role of NOS substrate in the relaxation responses, we incubated lung parenchymal strips from room air and hyperoxic groups with 10⁻⁴ M L-arginine or D-arginine for 30 min before preconditioning with bethanechol and subsequent EFS as already described. This concentration of L-arginine has been previously employed by us in comparable studies employing tracheal rings from rat described. This concentration of L-arginine has been previously employed by us in comparable studies employing tracheal rings from rat described. This concentration of L-arginine has been previously employed by us in comparable studies employing tracheal rings from rat described. This concentration of L-arginine has been previously employed by us in comparable studies employing tracheal rings from rat described. This concentration of L-arginine has been previously employed by us in comparable studies employing tracheal rings from rat described.

Guanosine 3,5'-cyclic monophosphate assay. Guanosine 3,5'-cyclic monophosphate (cGMP), a key intracellular second messenger molecule for NO signaling, was measured in acetylated samples through a competitive enzyme immunoassay (EIA) method using a cGMP EIA kit as per the manufacturer’s instruction (Cayman Chemicals, Ann Arbor, MI). Ultrapure water filtered through activated charcoal without organic impurities was used to dissolve all the reagents for this assay. The detection limit of the assay was 0.1 pmol/ml. For cGMP measurements, lung parenchymal strips from room air or hyperoxic rat pups were obtained, stabilized, and snap-frozen (n = 10 per group). Other sets of lung parenchymal strips from room air and hyperoxic pups (n = 6 per group) were incubated with l- or d-arginine (10⁻⁴ M) for 30 min after the stabilization and then snap-frozen. EFS was not applied to lung parenchymal strips used for cGMP measurements as EFS might utilize cGMP for relaxation purposes. Frozen lung parenchymal strips were thawed in chilled 5% trichloroacetic acid (TCA), homogenized (10% wt/vol), centrifuged, and supernatant was collected. TCA was extracted twice by 5 Vol of water-saturated ether. Residual ether was removed by heating samples at 70°C for 10 min. Five-hundred microliters of sample were acetylated by adding 100 µl of 4 M KOH and 25 µl of acetic anhydride, by vortexing for 15 s. Further 25 µl of 4 M KOH were added and samples were vortexed for 15 s. The standards were acetylated in the same way. Samples were then diluted to 1:50 and 1:100 dilutions and added to ELISA plates ( precoated with mouse monoclonal anti-arginine antibodies and blocked with blocking agent) along with cGMP-acetylcholinesterase conjugate and cGMP-specific rabbit antibodies for 18 h incubation at 4°C in parallel with the standard samples for the cGMP standard curve. After the incubation, the ELISA plate was washed to remove any unbound reagents and then the Ellman’s reagent (containing substrate for acetylcholinesterase) was added. The product of this enzymatic reaction was read at 405-nm wave length. The concentration of cGMP in the samples was calculated according to the standard curve and presented in picomoles per gram of tissue (weight).

Arginase assay. Arginase assay was performed in 12-day-old rat pups after 7 days exposure to room air (n = 10) or hyperoxia (n = 10). Animals were killed, lungs were removed, and the left lobe was snap-frozen in liquid nitrogen to determine arginase activity. The arginase activity was measured as described by Jenkinson and Grigor (20). In brief, lung tissues were thawed on ice in chilled storage buffer (10 mM Tris, pH 7.5, 10 mM glycine, 10 mM MnCl2, 10 mM 2-mercaptoethanol), homogenized in 4 Vol of storage buffer. The homogenate was centrifuged at 15,000 g for 15 min at 4°C, and supernatant was collected. Aliquots of supernatant (50 µl) were preactivated by addition of 10 µl of 10 mM MnCl2 followed by heating for 5 min at 55°C in a total volume of 100 µl. One-hundred microliters of 250 mM arginine, pH 9.7, was added to the preactivated samples, mixed, and the mixture was incubated at 37°C. Arginase reaction was stopped by addition of 1 ml of freshly prepared diacytetyl monoxime/acid solution prepared as follows: 1 ml of diacetyl monoxime reagent (3% diacetyl monoxime in 95% ethanol) was mixed with 29 ml of acid solution (270 ml concentrated H3PO4 plus 90 ml of concentrated H2SO4, made to 1 liter with water). The tubes were vortexed, sealed, and heated in a boiling water bath for 30 min. Tubes were cooled to room temperature and the urea concentration in each sample was measured with a spectrophotometer at 490 nm. Baseline urea concentration in each sample was also measured without incubation with L-arginine. The arginase activity was calculated as (sample urea concentration − baseline urea concentration)/10 and is expressed as nanomoles of urea per minute per milligram of protein. Urea samples containing 10, 50, and 90 µl of 10 mM urea (0.1, 0.5, and 0.9 µl) were incubated with each batch of assay samples. One unit of arginase activity is equivalent to the conversion of 1 µmol urea/min at 37°C. The protein content of tissue samples was determined by the modified method of Bradford (9) using Bio-Rad RC DC protein assay kit. Bovine serum albumin was used as the standard.

Wet-to-dry weight ratio. Additional rat pups (room air, n = 11 and hyperoxia, n = 12) were killed at 12 days to determine wet-to-dry lung ratios. A piece of the right lower lobe of lung was blotted to remove excess blood, placed on preweighed aluminum foil, and weighed (wet weight). The lungs were then completely dried at 60°C for 10 days and reweighed (dry weight). Wet weights were normalized and the dry weights were compared between room air and hyperoxic groups.

Statistical analysis. The results are expressed as means ± SE. Statistical significance was determined by two-way ANOVA with repeated measurements to determine the effect of EFS on relaxation responses between ages and hyperoxia vs. room air groups. To
analyze differences between individual voltages, post hoc comparison via Tukey-Kramer-multiple comparison test was used. For the measurements of cGMP and arginase activity, Student’s t-test was used. In all cases, P < 0.05 was considered statistically significant.

RESULTS

Effects of postnatal maturation on the relaxation responses to EFS. To evaluate maturational changes, we studied the relaxation of lung parenchymal strips of room air- or hyperoxia-exposed rat pups at different postnatal ages (day 7, 12, and 21). As shown in Fig. 1A, a greater relaxation was observed in less mature rat pups (7 and 12 days) vs. more mature rat pups (21 days) from room air groups in response to EFS (P < 0.01). There was no difference between relaxation responses at 7 and 12 days of age. There was a significantly greater relaxation at 50 V of EFS at both 7 and 12 days compared with 21 days (P < 0.01) for room air-exposed rat pups. We did not observe any difference in percent relaxation at different ages for hyperoxic groups (Fig. 1B).

Effect of hyperoxia on EFS-induced relaxation of lung parenchymal strips. We compared the relaxation responses between room air and hyperoxic groups at each age. There was a difference in responses between room air and hyperoxic groups at 7 (P < 0.001) and 12 (P < 0.001) days but not at 21 days of age. As expected, the relaxation response in all animals of all groups increased significantly at higher voltages. In the hyperoxic group at age 7 days, EFS-induced relaxation was reduced significantly at 45 V (P < 0.05) and at 50 V (P < 0.001) compared with the room air group (Fig. 2A). At age 12 days, relaxation was reduced significantly in hyperoxia-exposed animals, at 40 V (P < 0.05), 45 V (P < 0.001), and 50 V (P < 0.001), compared with room air-exposed animals (Fig. 2B). There were no significant differences between hyperoxic and room air animals at age 21 days (Fig. 2C).

Effect of NOS blockade on relaxation of lung parenchymal strips. NOS blockade was used in pups studied at day 12 to determine whether NO signaling is involved in EFS-induced relaxation responses after room air and hyperoxia exposure. EFS-induced relaxation was reduced significantly after the addition of L-NAME to the tissue bath containing lung parenchymal strips from room air animals (P < 0.001). This relaxation was significantly reduced both at 45 V (P < 0.05) and at 50 V (P < 0.001; Fig. 3A). L-NAME did not produce any change in relaxation of lung parenchymal strips from hyperoxic animals (Fig. 3B).

Effect of L-arginine supplementation on relaxation of lung parenchymal strips. To define whether NOS substrate depletion during hyperoxia might diminish EFS-induced relaxation, the lung parenchymal strips from room air and hyperoxic pups were incubated with the NO precursor L-arginine before EFS was applied (n = 8 per group). In room air animals, L-arginine did not change the relaxation responses to EFS (Fig. 4A), whereas in hyperoxic animals EFS increased relaxation induced by EFS (P < 0.05; Fig. 4B). This effect of L-arginine was significant at 40, 45, and 50 V (all P < 0.05) in the hyperoxic group. D-Arginine, the metabolically inactive isoform of L-arginine, did not induce any significant changes either in room air or hyperoxic groups (Fig. 4).

cGMP measurements. In separate but comparably prepared lung parenchymal strips, cGMP was measured to assess the generation of biologically active NO in these tissues from room air- and hyperoxia-exposed rat pups (n = 8 per group). In control strips from room air animals, the concentration of cGMP was significantly higher (20.60 ± 1.37 pmol/g, n = 10) compared with the hyperoxic group (7.95 ± 1.35 pmol/g, P < 0.001, n = 10). After normalization, the dried weight of lungs from hyperoxic animals was 2% less than the dry weight from room air animals. When lung parenchymal strips from hyperoxic animals were incubated with L-arginine, cGMP levels were significantly increased to 27.57 ± 3.8 pmol/g (n = 6, P < 0.001) compared with hyperoxic controls. In contrast, the addition of L-arginine did not affect cGMP levels from room air animals (n = 6; Fig. 5).

Measurement of arginase activity. To define whether depletion of the NOS substrate L-arginine during hyperoxia might be due to increased arginase activity of lung tissue, lung parenchymal strips from room air (n = 10) and hyperoxic (n = 10) pups were prepared, incubated in a comparable manner, and arginase activity was measured. In hyperoxic lung strips, arginase activity was significantly (P < 0.05) increased to 43.2 ± 8.8 compared with 16.9 ± 6.1 nmol urea·min⁻¹·mg protein⁻¹ in room air lung strips (Fig. 6).
Despite the widespread problem of increased airway reactivity among pediatric patients, maturational studies of airway smooth muscle contractility have received relatively scant attention (13). Furthermore, ontogeny of airway and tissue relaxant properties has been only minimally investigated with somewhat conflicting findings regarding developmental changes (10, 12). We previously documented that preconstricted tracheal smooth muscle from piglets exhibits NO-mediated relaxation over the first 3 wk of life that is lost by 3 mo (19). The current study documented a similar phenomenon in early rat pup development employing lung parenchymal strips under in vitro conditions. The greater relaxant properties of this tissue in the first 2 wk of rat pup development demonstrate that this phenomenon occurs across different mammalian species. We speculate that any pathophysiological insult to this increased relaxant component during early postnatal life may enhance vulnerability to airway hyperreactivity.

We clearly documented that NO-cGMP signaling represents a major mechanism for the lung parenchymal strip relaxation we observed from room air-exposed rat pups. This is consistent with recently documented upregulation of the relaxant response of bronchial smooth muscle from rat pups vs. adult animals in response to NO donor administration, as well as upregulation of NO-stimulated soluble guanylate cyclase activity in newborn vs. adult bronchial tissue (3). Consistent with the potential vulnerability of this system, hyperoxic exposure...
clearly inhibited this NO-mediated relaxant response of lung tissue in our study. In our previous studies, we demonstrated that hyperoxic exposure increases the response of lung resistance to vagal stimulation in rat pups under in vivo conditions (17). Unlike normoxic controls, NOS blockade did not further increase the resistance response to vagal stimulation in the hyperoxia-exposed rat pups (17). We also observed that hyperoxic exposure impairs relaxation of tracheal cylinders under in vitro conditions; however, this appeared to be associated with hyperoxic inhibition of prostaglandin-cAMP rather than NO-cGMP signaling (30). Therefore, the logical next step was to perform comparable studies in lung parenchymal strips under in vitro conditions. The current observations confirm that hyperoxic exposure significantly impairs NO-mediated relaxant responses at this level of the developing respiratory system.

In vitro study of lung parenchymal strips has several advantages over in vivo study of lung function. These include the ability to characterize the behavior of distal peripheral airways under carefully controlled conditions in the absence of circulating elements and centrally mediated neuronal components. The proportion of rat parenchymal strip occupied by alveolar wall, blood vessel, and airway is qualitatively similar to fractional areas in human lung strips (5). We do recognize that lung parenchymal strips contain various anatomic constituents including alveolar, bronchial, and blood vessel walls, as well as connective tissue and interstitial cells with varying contractile properties (14). However, rodent parenchymal strips have been shown to provide a useful measure of actual tissue mechanical properties (14). Future studies might correlate these in vitro findings with in vivo techniques employing low-frequency forced oscillation to partition respiratory system mechanics (8). Such novel in vivo techniques will be needed to translate the relevance of our in vitro findings to in vivo airway hyperreactivity induced by hyperoxic exposure. In this regard, it is encouraging that our results do parallel those in isolated tra-

Fig. 4. Effect of l-arginine (l-Arg) supplementation on relaxation of lung parenchymal strips. A: l-arginine did not change EFS-induced relaxation of lung parenchymal strips in 12-day-old room air animals. B: in hyperoxic animals, addition of l-arginine increased relaxation compared with hyperoxic controls (P < 0.05); however, a biologically inert d-arginine (d-Arg) did not produce any changes in any group. †P < 0.05 vs. hyperoxic controls.

Fig. 5. Levels of guanosine 3’,5’-cyclic monophosphate (cGMP) in lung parenchymal strips were lower in hyperoxic vs. room air-exposed rat pups at 12 days (P < 0.001). After addition of l-arginine, cGMP levels increased significantly in hyperoxic rat pups (P < 0.001). RA, room air (n = 10); H, hyperoxia (n = 10); RA + l-Arg, room air + l-arginine (n = 6); H + l-Arg, hyperoxia + l-arginine (n = 6).

Fig. 6. Arginase activity in lung parenchymal strips was higher in hyperoxic vs. room air-exposed rat pups at 12 days (P < 0.05).
Various animal models have been employed to characterize the mechanisms underlying disruption of NO-cGMP signaling during neonatal lung injury. The effects of lung injury on pulmonary NOS expression have been inconsistent in immature animal models, possibly related to interspecies differences and various approaches to inducing lung injury. While chronically ventilated preterm baboons and lambs exhibited a decline in NOS isoforms compared with controls (1, 25), hyperoxic exposure enhanced NOS expression in whole lung and fetal pulmonary artery endothelial cells (33, 36). We, therefore, sought to focus our studies on NO-cGMP signaling by measurement of cGMP levels under conditions of room air and hyperoxic exposure. Consistent with our physiological findings, we documented a significant decrease in cGMP levels after hyperoxic exposure. In view of this clear decrease in cGMP levels, without evidence of decreased NOS expression from prior studies (33, 36), we hypothesized that depletion of the NO precursor, L-arginine, might occur under hyperoxic conditions.

To investigate whether hyperoxia depleted L-arginine, contributed in the reduction of relaxation of lung parenchymal strips, we provided exogenous L-arginine to lung parenchymal strips. We observed that the addition of exogenous L-arginine restored the relaxation of preconstricted lung parenchymal strips to EFS in hyperoxia-exposed animals. These data suggest that L-arginine was depleted from the tissues due to hyperoxia, becoming a limiting factor for NO production under these conditions. This is consistent with our own prior data in a homozygous cystic fibrosis transmembrane conductance regulator negative mouse model of cystic fibrosis in which we demonstrated that impaired relaxation of isolated tracheas can be reversed with L-arginine supplementation (29). While L-arginine likely reverses impaired relaxation by enhancing NO-cGMP signaling, a potential role of L-arginine in formation of nitrosoglutathione or other nitrosocompounds by other pathways cannot be excluded (27). To test that the effect of L-arginine was specific, we used D-arginine, the biologically inert amino acid, as control. Exogenous D-arginine did not change the relaxant responses in either room air or hyperoxic groups. These data are consistent with those of de Boer et al. (11) who demonstrated that deficiency of endogenous NO precursor, L-arginine, might occur under hyperoxic conditions.

1-L-Arginine can be metabolized by NOS to produce NO and L-citrulline, or by arginase to produce urea and L-ornithine (32). Therefore, NOS and arginase are in competition for a common substrate, L-arginine. The enzyme arginase, which converts L-arginine to urea and L-ornithine, may affect NOS activities by decreasing L-arginine bioavailability through this competition. It has been shown that hyperoxia increases arginase activity in serum (31), liver (26), and lung (37). Morris et al. (31) showed that in asthmatic patients serum arginase activity was elevated compared with controls. Furthermore, Malleske et al. (26) showed that hyperoxia diminishes NO production in mice due to increase in hepatic arginase I activity. Que et al. (37) also showed upregulation of expression of arginase in connective tissue suggesting that the function of pulmonary arginase may be linked to connective tissue elements, e.g., fibroblasts, during lung injury and recovery. Consistent with these earlier observations, we also found increased arginase activity in lung strips from hyperoxia-exposed rat pups compared with room air controls suggesting the depletion of substrate for NO production in hyperoxic lung tissue. Conversion of L-arginine to L-ornithine, a precursor for proline synthesis, may also contribute to the diminished relaxant properties of hyperoxic lung tissue. Furthermore, it is known that alveolar epithelial cells undergo phenotypic transition to myofibroblasts through a process of epithelial-mesenchymal transition (41) and NO has been shown to attenuate epithelial-mesenchymal transition in alveolar epithelial cells (38). Of particular interest is the recent observation that bronchial epithelial cells from mice are capable of such epithelial-mesenchymal transition (40). A decrease in bioavailability of L-arginine and NO might therefore decrease relaxation responses due to increased mass of myofibroblasts in hyperoxia-exposed tissues.

In addition to the decrease in bioavailability of L-arginine under hyperoxic conditions, we cannot exclude a role for reactive oxygen species such as superoxide and peroxynitrite decreasing NO activity in our studies, as has been reported in animal models focused on pulmonary hypertension (34). The cofactor tetrahydrobiopterin (BH4) is an important regulator of NOS enzymatic activity. Without BH4, NO production is reduced and superoxide production is increased as a result of the loss of enzymatic coupling between the reduction in molecular oxygen and oxidation of L-arginine (6, 23). The possibility of decreased levels of BH4 due to increased reactive oxygen-dependent consumption of NO and reduced NOS activity also cannot be excluded.

Several additional limitations must be considered in interpretation of our findings regarding impaired NO-cGMP signaling in the hyperoxia-exposed rat pup model. While we observed comparable physiological effects in 7- and 12-day-old rat pups, subsequent mechanistic studies related to arginine depletion and increased arginase activity were only performed at 12 days. It is certainly possible that mechanisms of oxidant injury and their effects on NO biology alter over this period of maturation. As noted in MATERIALS AND METHODS, some experiments were performed 24 h after the supplemental oxygen had been discontinued and this may have affected oxidant-related mechanisms. However, no apparent differences in physiological responses were noted in relation to timing of discontinuation of oxygen exposure suggesting a prolonged effect of byproducts formed due to oxidant injury. Another potential factor that might have influenced our results was the use of wet lung weight to normalize cGMP measurements in both room air- and hyperoxia-exposed rat pups. However, in a subsequent set of comparably exposed animals, we observed only a modest decrease in dry weight after 7 days of hyperoxic vs. room air exposure, making this an unlikely confounder of our results. In this study, we have not characterized the expression and activity of the various NOS isoforms under control and experimental conditions, but rather focused on downstream signaling via the NO-cGMP pathway. However, future studies need to address this issue which is beyond the scope of the current study.

In summary, we documented a significant reduction in the relaxation of lung parenchymal strips from neonatal rat pups exposed to hyperoxia. We implicated disrupted NO-cGMP signaling pathways as a potential contributor to the hyperoxic lung injury phenomenon.
signaling as a major contributor to this phenomenon, via depletion of the substrate L-arginine. We speculate that this mechanism might play a role in the increased airway hyperactivity observed in human infants exposed to high O₂ concentration. This is consistent with emerging clinical data that supplementation of preterm infants with inhaled NO is associated with decreased need for bronchodilators and inhaled steroid therapy at 12 mo of age (16). A role for arginine supplementation as an alternative approach to inhaled NO might be worthy of study in preterm human infants.

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