Moderate postnatal hyperoxia accelerates lung growth and attenuates pulmonary hypertension in infant rats after exposure to intra-amniotic endotoxin

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Tang J, Seedorf GJ, Muehlthaler V, Walker DL, Markham NE, Balasubramaniam V, Abman SH. Moderate postnatal hyperoxia accelerates lung growth and attenuates pulmonary hypertension in infant rats after exposure to intra-amniotic endotoxin. Am J Physiol Lung Cell Mol Physiol 299: L735–L748, 2010. First published August 13, 2010; doi:10.1152/ajplung.00153.2010.—To determine the separate and interactive effects of fetal inflammation and neonatal hyperoxia on the developing lung, we hypothesized that: 1) antenatal endotoxin (ETX) causes sustained abnormalities of infant lung structure; and 2) postnatal hyperoxia augments the adverse effects of antenatal ETX on infant lung growth. Escherichia coli ETX or saline (SA) was injected into amniotic sacs in pregnant Sprague-Dawley rats at 20 days of gestation. Pups were delivered 2 days later and raised in room air (RA) or moderate hyperoxia (O2, 80% O2 at Denver’s altitude, ~65% O2 at sea level) from birth through 14 days of age. Heart and lung tissues were harvested for measurements. Intra-amniotic ETX caused right ventricular hypertrophy (RVH) and decreased lung vascular endothelial growth factor (VEGF) and VEGF receptor-2 (VEGFR-2) protein contents at birth. In ETX-exposed rats (ETX-RA), alveolarization and vessel density were decreased, pulmonary vascular wall thickness percentage was increased, and RVH was persistent throughout the study period compared with controls (SA-RA). After antenatal ETX, moderate hyperoxia increased lung VEGF and VEGFR-2 protein contents in ETX-O2 rats and improved their alveolar and vascular structure and RVH compared with ETX-RA rats. In contrast, severe hyperoxia (≈95% O2 at Denver’s altitude) further reduced lung vessel density after intra-amniotic ETX exposure. We conclude that intra-amniotic ETX induces fetal pulmonary hypertension and causes persistent abnormalities of lung structure with sustained pulmonary hypertension in infant rats. Moreover, moderate postnatal hyperoxia after antenatal ETX restores lung growth and prevents pulmonary hypertension during infancy. Antenatal inflammation; vascular endothelial growth factor; bronchopulmonary dysplasia; persistent pulmonary hypertension of the newborn

Antenatal inflammation is the most common identifiable cause of preterm labor (19), and intrauterine infection is present in most cases of preterm birth before 30 wk of gestation (8, 18, 46). Multiple clinical studies have shown that chorioamnionitis is strongly linked with an increased risk for the development of bronchopulmonary dysplasia (BPD) (14, 22, 40, 41, 45, 48, 53, 64, 66), which is the chronic lung disease of infancy that follows premature birth and injury to the immature lung. Despite improvements in perinatal care, BPD remains one of the most significant sequelae of prematurity (5). Clinical practices that attempt to reduce postnatal risk factors for BPD have been vigorously applied to the care of premature newborns, but little is known about how to protect neonatal lung growth in the setting of antenatal inflammation. The development of innovative protective strategies requires a greater understanding of mechanisms through which normal lung development is disrupted by inflammatory stimuli in utero.

Although early studies showed strong associations of chorioamnionitis with BPD, more recent clinical studies have failed to demonstrate an increase in the incidence of BPD in premature infants who were exposed to chorioamnionitis (3, 15, 28, 29, 33, 50, 52, 67). Disrupted lung development has been demonstrated in animal models of chorioamnionitis (9, 20, 44, 61, 68), but other experimental studies showed that intra-amniotic inflammatory stimuli do not necessarily impair lung structure when assessed later during infancy (43, 47). It has been suggested that the postnatal environment may interact with antenatal inflammation to further worsen late respiratory outcomes of preterm infants exposed to chorioamnionitis. For example, clinical data suggest that chorioamnionitis is not associated with an increased risk of BPD in the absence of neonatal sepsis or prolonged mechanical ventilation in at-risk premature infants (62). Moreover, postnatal inflammatory stimuli, such as severe hyperoxia, may amplify the negative impacts of antenatal inflammation on neonatal lung growth in rodent models (9, 47, 63). Oxygen therapy is frequently required for respiratory support of premature newborns after exposure to antenatal inflammatory stimuli, but whether postnatal hyperoxia potentiates abnormalities of lung structure after chorioamnionitis is unclear. In addition, variability in the risk for BPD in preterm infants exposed to various levels of hyperoxia remains unexplained (35).

Past studies have shown that vascular endothelial growth factor (VEGF) plays a critical role in regulating vascular and distal air space growth during lung development (10, 16, 17). Disruption of VEGF signaling impairs lung growth in neonatal rats (24, 36, 39, 59, 60), and downregulation of lung VEGF is demonstrated in experimental models of BPD with postnatal hyperoxia (23, 37, 38, 60) and with antenatal inflammation (25). Whether hyperoxia would further suppress lung VEGF expression after antenatal inflammation has not been studied.

Thus, to determine the separate and interactive effects of fetal inflammation and postnatal hyperoxia on the developing lung, we hypothesized that antenatal endotoxin (ETX) causes sustained abnormalities of infant lung structure; and 2) postnatal hyperoxia augments the adverse effects of antenatal ETX on infant lung growth.
impairment of lung structure during infancy. We further proposed that postnatal hyperoxia augments the adverse effects of antenatal ETX on infant lung structure. We report that intra-amniotic ETX caused signs of pulmonary hypertension at birth, increased mortality, and impaired lung structure in neonatal rats. In surviving rat pups exposed to antenatal ETX, abnormalities of lung structure and evidence of pulmonary hypertension persisted through the first 2 wk of life. In addition, rather than worsening lung structure, we found that treatment of ETX-exposed rat pups with moderate postnatal hyperoxia enhanced lung growth and improved pulmonary hypertension during infancy. Moreover, lung VEGF and VEGFR-2 protein contents were decreased at birth in ETX-exposed rats but were upregulated during moderate postnatal hyperoxia in infant rats after antenatal ETX exposure. Overall, these findings suggest that antenatal inflammation may be sufficient to impair infant lung structure and cause pulmonary hypertension at birth and during infancy, but rather than potentiating lung injury, moderate postnatal hyperoxia restores lung structure and attenuates pulmonary hypertension after antenatal ETX.

MATERIALS AND METHODS

Animals

All procedures and protocols were approved by the Animal Care and Use Committee at the University of Colorado Health Sciences Center. Pregnant Sprague-Dawley rats were purchased from Charles River Laboratories (Wilmington, MA) and maintained in room air at Denver’s altitude (1,600 m; barometric pressure, 630 mmHg; inspired oxygen tension, 122 mmHg) for at least 1 wk before giving birth. Animals were fed ad libitum and exposed to day-night cycles alternately every 12 h. Rats were killed with an intraperitoneal injection of pentobarbital sodium (0.3 mg/g body wt; Fort Dodge Animal Health, Fort Dodge, IA).

Study Design

Intra-amniotic ETX administration. At 20 days gestation (term: 22 days), pregnant rats were prepared for receiving intra-amniotic injections. The timing of injection during the late canalicular stage of lung development in the rat was selected to parallel the similar stage of human lung development in 24- to 26-wk premature newborns, who are at the highest risk for BPD. After premedication with buprenorphine (0.01–0.05 mg/kg, subcutaneous injection), laparotomy was performed in pregnant rats under general anesthesia with 1–2% isoflurane inhalation via face mask (anesthesia machine: Matrix by Midmark, model VIP3000). During anesthesia and laparotomy, pregnant rats were kept on a heating pad for preventing hypothermia. Pregnant rats were randomly assigned to saline control (SA) or endotoxin (ETX) group; the SA group received 50 μl of normal saline per amniotic sac, and the ETX group received 10 μg of Escherichia coli 055:B55 ETX (Sigma Chemical, St. Louis, MO) diluted to 50 μl with normal saline per sac. Under sterile preparation, a midline abdominal incision of 3–4 cm in length was made to expose the amniotic sacs for intra-amniotic injections. The amniotic sac closest to the right ovary was first identified and injected, and then in a counterclockwise sequence each sac was identified and injected with a maximum of 10 sacs injected per dam. ETX injections were limited to 10 sacs to prevent maternal mortality due to systemic toxicities from accumulating doses of intra-amniotic ETX. The total number of amniotic sacs in each mother rat was examined and recorded during laparotomy. We established the dose of ETX from preliminary studies in which ETX at lower doses (1–5 μg/sac) failed to induce abnormal lung structure at 14 days of age. The abdominal incision was closed with nylon sutures. Bupivacaine (1–2 mg/kg, intramuscular injection) was applied over the incision wound for postoperative pain control. Pregnant rats were monitored closely to ensure arousal within 10 min after surgery, and rats were placed back to the cages and were monitored for activity, ability to drink and eat, and for signs of bleeding or infection.

Cesarean section. Two days after intra-amniotic injections, cesarean section was performed on pregnant rats under general anesthesia with isoflurane inhalation, as described above. The fetus in the amniotic sac closest to the right ovary was first delivered, which was followed by delivery of the rest of the fetuses in a counterclockwise sequence, to identify fetuses exposed to intra-amniotic injections. The total number of amniotic sacs in each mother rat was further verified at the time of delivery. The main reason for performing cesarean section instead of allowing vaginal delivery is to identify the fetuses exposed to intra-amniotic injections, based on the order of the amniotic sacs and their anatomic locations related to the ovaries. In addition, preliminary studies showed decreased perinatal mortality after cesarean section compared with vaginal delivery. All of the rat pups in the injected amniotic sacs were delivered within 5 min after onset of anesthesia. Mother rats were then euthanized with pentobarbital sodium. Newborn rats were immediately placed on a heating pad to avoid hypothermia and were dried manually with gauze sponges. Pups received no supplemental oxygen or artificial ventilation at birth. Survival rate at birth was recorded. Within 30 min after birth, the pups were weighed and placed with foster mother rats in regular cages, to be raised in room air or hyperoxia chambers through 14 days of age, as randomly assigned. Hyperoxia exposure is described below.

Hyperoxia exposure. Pups assigned to hyperoxia were placed into hyperoxia chambers, along with the foster mother rats, within 30 min after birth. The oxygen concentration in the chambers was maintained at FIO₂ = 0.80 (PO₂ = 466 mmHg at Denver’s altitude with barometric pressure = 630 mmHg, which is equivalent to an FIO₂ of 0.65 at sea level) for 14 days. Hyperoxia exposure was continuous with only brief interruptions for animal care (less than 10 min/day). The concentration of oxygen was controlled by the use of a ProOx (Remington Bioinstruments). To determine the differential effects between moderate and severe hyperoxia, high oxygen concentration at FIO₂ = 0.95 (equivalent to FIO₂ of 0.75–0.80 at sea level) was also performed as a parallel protocol. Dams were rotated during ≥95% oxygen exposure due to poor tolerance of adult rats to severe hyperoxia.

Rat lungs were harvested at birth and 5 days of age for Western blot analysis, and at 7 and 14 days of age for histological assessment. Hearts were dissected and weighed at birth and 2, 7, and 14 days of age. Three to eight rats were studied in each group for each measurement at each time point. Survival of the infant rats was monitored and recorded daily from birth throughout the study period. Survival rate was calculated as the number of surviving pups divided by the number of sacs that received intra-amniotic injection in each given litter. Body weight was measured at birth and at the time of being killed for study measurements.

Tissue for histological analysis. Animals were killed with intraperitoneal pentobarbital sodium. A catheter was placed in the trachea, and the lungs were inflated with 4% paraformaldehyde and maintained at 20 cm H₂O pressure for 60 min. A ligature was tightened around the trachea to maintain pressure, and then the tracheal cannula was removed. Lungs were then immersed in 4% paraformaldehyde at room temperature overnight for fixation. A 2-mm thick transverse section was taken from the mid-plane of right lower lobe and left lobe of the fixed lungs per animal, respectively. Two sections from each animal were processed and embedded in paraffin wax.

Immunohistochemistry

Slides with 5-μm paraffin sections were stained with hematoxylin and eosin for assessing alveolar structures and with von Willebrand Factor (vWF), an endothelial cell-specific marker, for assessing vascular density and vascular wall thickness.
Morphometric Analysis

Radial alveolar counts (RAC), mean linear intercept (MLI), and other indices of alveolar structure, and pulmonary vessel density and vascular wall thickness, were determined by standard morphometric techniques, as outlined below. In each animal, at least five measurements were obtained for RAC, at least 10 images were processed for computer-assisted image analysis of alveolar structure, and at least 10 pulmonary vessels were measured for pulmonary vessel density and vascular wall thickness.

RAC. Alveolarization was assessed by the RAC method of Emery and Mithal as described (11, 12). Respiratory bronchioles were identified as bronchioles lined by epithelium in one part of the wall. From the center of the respiratory bronchiole, a perpendicular line was dropped to the edge of the acinus connective tissues or septum or pleura, and the number of septae intersected by this line was counted.

MLI and other indices of alveolar structure. Measurements of MLI, internal lung surface area, nodal point density, and interstitial thickness were performed with a computer-assisted image analysis program. Briefly, the images were captured on a Zeiss Axioscope2 microscope, using the ×10 objective and captured as a high-resolution TIFF image by a MicroPublisher digital camera (2,560 × 1,940 pixel resolution; Qimaging, Burnaby, Canada). The fields with large airways or vessels were avoided. These images were processed with a plug-in that was previously developed by Dr. V. Balasubramanian (4) and Dr. C. Coulon to use ImageJ, a public domain Java image-processing program created by Wayne Rasband at the Research Services Branch, National Institute of Mental Health, Bethesda, MD (http://rsb.info.nih.gov/ij).

Pulmonary vessel density. Pulmonary vessel density was determined by counting vWF-stained vessels with external diameter less than 100 μm per high-power field. The fields containing large airways or vessels were avoided.

Pulmonary vascular wall thickness percentage. Wall thickness of pulmonary vessels was measured on those with external diameter at 10–30 μm in size. This range of vascular size was chosen based on the preliminary observations that the smallest pulmonary vessels showed the most remarkable remodeling after exposure to intra-amniotic ETX. Wall thickness and external diameter were measured

Fig. 1. Effects of intra-amniotic antenatal endotoxin (ETX) and moderate postnatal hyperoxia on survival rate of infant rats. As shown, survival in the ETX-RA group was worse than SA-RA controls (P < 0.05 ETX-RA vs. SA-RA). SA-RA, saline-room air; ETX-RA, endotoxin-room air; SA-O₂, saline-80% O₂; ETX-O₂, endotoxin-80% O₂.

Fig. 2. A–C: effects of intra-amniotic ETX and moderate postnatal hyperoxia on body weight of infant rats. As shown, body weight was decreased by ETX compared with saline controls from birth through 14 days of age (P < 0.05, ETX-RA vs. SA-RA), and ETX-O₂ rats had higher body weights than ETX-RA rats at and after day 2 (P < 0.05).
under Adobe Photoshop CS. The percent medial thickness of an individual vessel was calculated by the following formula: (medial thickness × 2 × 100)/external diameter.

Right Ventricular Hypertrophy

The right ventricle (RV) and left ventricle plus septum (LV+S) were dissected and weighed, and the ratio of RV to LV+S weights was determined.

Oxygen Saturation

Oxygen saturation (SatO2) was measured by using Nonin Pulse Oximeter (Nonin Medical, Plymouth, MN) on rats raised in room air. During measurement, animals were kept on a heating pad to avoid hypothermia. No sedatives were given during this measurement to avoid hypventilation. For rats younger than 7–10 days of age or with weight less than 20 g, each end of the sensor was attached to each side of the cheeks. For older or bigger rats, the sensor was attached to the precordial site, and animals were placed in a prone position to keep good attachment to the sensor. The readings of SatO2 were interpreted as valid when the heart rate, which was simultaneously monitored on Nonin Pulse Oximeter, stably maintained within the age-appropriate range. Each measurement was finished within 10–15 s to avoid desaturation secondary to prolonged measurement. For each animal, three to five measurements were taken, and the highest value of SatO2 was used for analysis.

Western Blot Analysis

Frozen lung samples were homogenized in ice-cold buffer containing 50 mM Tris-HCl (pH 7.4), EDTA (1 mM), EGTA (1 mM), 0.1% 2-mercaptoethanol, 4-(2-aminoethyl)-benzenesulfonyl fluoride (1 mM), leupeptin (1 μM), and pepstatin A (1 μM). Samples were centrifuged at 1,500 g for 20 min at 4°C to remove cellular debris. Protein content in the supernatant was determined by the Bradford method (6), using BSA as the standard. Briefly, 25 μg of protein sample per lane was resolved by SDS-PAGE, and proteins from the gel were transferred to PVDF membrane. Blots were blocked overnight in 5% nonfat dry milk in PBS with 0.1% Tween 20. These blots were incubated for 1 h at room temperature with either rabbit anti-human polyclonal VEGF antibody (sc-507, 1:500; Santa Cruz Biotechnology), rabbit anti-human polyclonal VEGFR-2 antibody (KDR/flk-1, sc-504, 1:500; Santa Cruz Biotechnology), or mouse anti-human polyclonal eNOS (bd610297, 1:500; BD Biosciences) in 5% nonfat dry milk in PBS with 0.1% Tween 20. Blots were then incubated for 1 h at room temperature with a goat anti-rabbit IgG horseradish peroxidase (HRP) antibody (sc-2054, 1:10,000–1:40,000; Santa Cruz Biotechnology) or goat anti-mouse IgG HRP antibody (AP124P, 1:20,000; Chemicon). After being washed, bands were visualized by enhanced chemiluminescence (ECL Advance kit; Amersham Pharma- cia Biotech, Buckinghamshire, UK). For Western analysis of VEGF, recombinant mouse VEGF protein (Santa Cruz Biotechnology) was used as a control.

Fig. 3. Effects of intra-amniotic ETX and moderate postnatal hyperoxia on distal lung growth at days 7 and 14. A and C: lung micrographs are representative for each group and were obtained at the same magnification. Internal scale bar, 100 μm. B: at day 7, radial alveolar counts (RAC) were decreased in ETX-RA rats compared with SA-RA controls (P < 0.05), and ETX-O2 rats had higher RAC than ETX-RA rats (P < 0.05). D: at day 14, RAC remained decreased in ETX-RA rats compared with SA-RA controls and with ETX-O2 rats (P < 0.001 for each comparison).
used as a control. On each blot, β-actin was detected as a housekeeping protein to compare expression between samples. Mouse monoclonal β-actin antibody (Sigma A-5316; 1:10,000; Sigma-Aldrich) was the primary antibody used for β-actin. Densitometry was performed using ImageJ (v1.33q). Four to five animals were analyzed per study group.

Statistical Analysis

Statistical analysis was performed with the InStat 3.0 software package (GraphPad Software, San Diego, CA). Statistical comparison was made by analysis of variance and Fisher protected least significant difference test. Data are presented as means ± SE. P < 0.05 was considered significant.

RESULTS

Survival Rate and Body Weight

A total of nine litters of rat pups received intra-amniotic saline injections, and, after birth, six litters were raised in room air (SA-RA) and three litters were treated with 80% O2 (SA-O2). Fifteen litters of rat pups received intra-amniotic

<table>
<thead>
<tr>
<th>Group</th>
<th>MLI, mm</th>
<th>Internal Surface Area, mm²/HPF</th>
<th>Nodal Point Density, per HPF</th>
<th>Interstitial Thickness, mm</th>
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<tbody>
<tr>
<td>SA-RA (5)</td>
<td>55.4 ± 0.8</td>
<td>29,060 ± 496</td>
<td>1,632 ± 50</td>
<td>6.2 ± 0.1</td>
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<tr>
<td>ETX-RA (7)</td>
<td>73.6 ± 1.3†*</td>
<td>23,606 ± 516†*</td>
<td>1,164 ± 25†*</td>
<td>8.5 ± 0.8†</td>
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<tr>
<td>SA-O2 (5)</td>
<td>61.5 ± 2.2</td>
<td>28,737 ± 821</td>
<td>1,529 ± 63</td>
<td>6.5 ± 0.2</td>
</tr>
<tr>
<td>ETX-O2 (8)</td>
<td>55.5 ± 2.0</td>
<td>30,785 ± 970</td>
<td>1,878 ± 100</td>
<td>7.1 ± 0.2</td>
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Values are means ± SE. Number of animals in each group is in parentheses. SA-RA, saline-room air; ETX-RA, endotoxin-room air; SA-O2, saline-80% O2; ETX-O2, endotoxin-80% O2; MLI, mean linear intercept; HPF, high-power field. †P < 0.05 vs. SA-RA. *P < 0.001 ETX-RA vs. ETX-O2.

Fig. 4. Effects of intra-amniotic ETX and moderate postnatal hyperoxia on pulmonary vessel density. A and C: representative findings are shown for each group at days 7 and 14 after immunostaining for von Willebrand Factor (vWF). Micrographs were obtained at the same magnification. Internal scale bar, 100 μm. B: at day 7, pulmonary vessel density was decreased in ETX-RA rats compared with SA-RA controls and ETX-O2 (P < 0.001 for each comparison). D: at day 14, pulmonary vessel density in ETX-RA rats remained decreased when compared with SA-RA controls and ETX-O2 (P < 0.001 for each comparison).
ETX; 10 litters were raised in room air (ETX-RA), and 5 litters in 80% O2 (ETX-O2). Survival was recorded daily after birth for each group (Fig. 1). After receiving intra-amniotic ETX, 89% of rat pups survived at birth compared with 99% in saline controls \((P = 0.18)\). Survival rate of ETX-RA rats decreased to 59% and 57% at days 1 and 2, respectively, which was worse than survival in SA-RA controls \((P < 0.05, \text{comparisons at each age})\). With 80% O2, 85% of ETX-O2 pups survived at day 1 compared with 59% in ETX-RA group \(P = 0.11\). In 80% O2, 97% of SA-O2 group survived at day 1, which was not different from the other three groups. No mortality was found in any group at and beyond day 2.

Birth weight was decreased in ETX-exposed rats compared with saline controls \(P < 0.0001\; \text{Fig. 2}\). At postnatal day 2, ETX-RA rats continued to have body weights that were lower than SA-RA controls \(P < 0.05\), whereas ETX-O2 rats had higher body weights than ETX-RA rats \(P < 0.05\), which were comparable to SA-RA controls. At days 7 and 14 (Fig. 2), ETX-RA pups had persistent growth failure, as reflected by body weights below control values \(P < 0.05\) at each age). In contrast, ETX-O2 pups maintained higher body weights than ETX-RA pups at days 7 and 14 \(P < 0.05\) at each age), and growth was comparable to SA-RA controls. In saline controls, body weights of SA-O2 rats were similar to SA-RA rats at days 2 and 7, but fell slightly at day 14 \(P < 0.05\; \text{Fig. 2}\). There was no difference in body weights between ETX-O2 and SA-O2 rats at days 2, 7, and 14.

**Lung Histology and Morphometric Analysis**

Figure 3 shows the effects of intra-amniotic ETX and moderate postnatal hyperoxia on distal lung growth. At day 7 (Fig. 3B), RAC were decreased in ETX-RA rats compared with SA-RA controls \(P < 0.05\). ETX-O2 rats had higher RAC than ETX-RA rats at day 7 \(P < 0.05\), although were still reduced compared with SA-RA controls \(P < 0.05\). At day 14 (Fig. 3D), RAC remained decreased in ETX-RA rats compared with SA-RA controls \(P < 0.001\). RAC in ETX-O2 rats became comparable to SA-RA controls and continued being higher than ETX-RA rats at day 14 \(P < 0.001\). There was no difference in RAC between ETX-O2 and SA-O2 groups at either day 7 or day 14.

In comparison with SA-RA controls, ETX-RA rats had increased MLI \(P < 0.001\), decreased internal surface area \(P < 0.001\), decreased nodal point density \(P < 0.01\), and increased interstitial thickness \(P < 0.05\) (Table 1). Compared with ETX-RA rats, ETX-O2 rats had lower MLI \(P < 0.001\), higher internal surface area \(P < 0.001\), and higher nodal point density \(P < 0.001\), but there was no difference in interstitial thickness. Nodal point density was higher in ETX-O2 rats than in SA-O2 rats \(P < 0.05\); other parameters were not different between the ETX-O2 and SA-O2 groups.

The effects of intra-amniotic ETX and moderate postnatal hyperoxia on pulmonary vessel density are shown in Fig. 4. At day 7 (Fig. 4B), pulmonary vessel density was decreased in ETX-RA rats compared with SA-RA controls \(P < 0.001\);
ETX-O2 rats had higher vessel density than ETX-RA rats ($p < 0.001$). At this age, both ETX-O2 and SA-O2 groups had pulmonary vessel density comparable to SA-RA controls. At day 14 (Fig. 4D), pulmonary vessel density in ETX-RA rats remained decreased compared with SA-RA controls ($p < 0.001$); ETX-O2 rats continued to have higher vessel density than ETX-RA rats ($p < 0.001$), which was comparable to SA-RA controls. At this age, pulmonary vessel density in SA-O2 rats became lower than SA-RA controls ($p < 0.05$). There was no difference in vessel density between ETX-O2 and SA-O2 groups at either day 7 or day 14.

The effects of intra-amniotic ETX and moderate postnatal hyperoxia on pulmonary vascular wall thickness at day 14 are shown in Fig. 5. Pulmonary vascular wall thickness percentage increased in ETX-RA rats compared with SA-RA controls ($p < 0.001$). ETX-O2 rats had lower vascular wall thickness than ETX-RA rats ($p < 0.001$). Both ETX-O2 and SA-O2 groups were comparable to SA-RA controls. There was no difference between ETX-O2 and SA-O2 groups.

Changes in RAC and pulmonary vessel density from postnatal day 7 to day 14 are shown in Fig. 6. During the study period, RAC in ETX-O2 rats improved to similar values found in SA-RA controls, whereas pulmonary vessel density in SA-O2 rats was reduced to below measurements in SA-RA controls. In Fig. 6A, RAC were decreased in ETX-RA rats at day 7 ($p < 0.05$) and remained decreased at day 14 ($p < 0.05$) compared with SA-RA controls. ETX-O2 rats had higher RAC than ETX-RA rats at day 7 ($p < 0.05$) and remained higher at day 14 ($p < 0.05$). RAC in ETX-O2 rats were lower than SA-RA controls at day 7 ($p < 0.05$), midway through the hyperoxia exposure period, and then became comparable to SA-RA controls at the end of hyperoxia exposure. SA-O2 rats had similar RAC as measured in the SA-RA controls at days 7 and 14. In Fig. 6B, pulmonary vessel density decreased in ETX-RA rats at day 7 ($p < 0.05$) and remained decreased at day 14 ($p < 0.05$) compared with SA-RA controls. ETX-O2 rats had higher vessel density than ETX-RA rats at day 7 ($p < 0.001$) and remained higher at day 14 ($p < 0.001$). Both ETX-O2 and SA-O2 groups were comparable to SA-RA controls at day 14 ($p < 0.05$). Moderate hyperoxia reduced RV/LV+S ratio in ETX-O2 rats by day 2 compared with ETX-RA rats ($p < 0.001$).

Right Ventricular Hypertrophy

The effects of intra-amniotic ETX and moderate postnatal hyperoxia on the RV/LV+S ratio in infant rats are shown in Fig. 7. Effects of intra-amniotic ETX and moderate postnatal hyperoxia on the ratio of right ventricle to left ventricle plus septum weights (RV/LV+S) in infant rats. The RV/LV+S ratio was increased in ETX rats at birth compared with saline controls ($p < 0.05$; A) and remained elevated in ETX-RA rats throughout the study period ($p < 0.05$ for each age; B). Moderate hyperoxia reduced RV/LV+S ratio in ETX-O2 rats by day 2 compared with ETX-RA rats ($p < 0.001$).

Fig. 6. Changes in RAC and pulmonary vessel density from postnatal day 7 to day 14. A: RAC were decreased in ETX-RA rats at day 7 ($p < 0.05$) and remained decreased at day 14 ($p < 0.05$) compared with SA-RA controls and ETX-O2 rats ($p < 0.05$ for each comparison). B: pulmonary vessel density was decreased in ETX-RA rats at day 7 ($p < 0.05$) and remained decreased at day 14 ($p < 0.05$) compared with SA-RA controls. ETX-O2 rats had higher vessel density than ETX-RA rats at days 7 and 14 ($p < 0.001$ for each age).

Fig. 7. Effects of intra-amniotic ETX and moderate postnatal hyperoxia on the ratio of right ventricle to left ventricle plus septum weights (RV/LV+S) in infant rats. The RV/LV+S ratio was increased in ETX rats at birth compared with saline controls ($p < 0.05$; A) and remained elevated in ETX-RA rats throughout the study period ($p < 0.05$ for each age; B). Moderate hyperoxia reduced RV/LV+S ratio in ETX-O2 rats by day 2 compared with ETX-RA rats ($p < 0.001$).
Fig. 7. The RV/LV+S ratio increased in ETX-exposed rats at birth compared with saline controls ($P < 0.05$) and remained elevated in ETX-RA rats throughout the study period compared with SA-RA controls ($P < 0.05$ for each age). Moderate hyperoxia reduced the RV/LV+S ratio in ETX-O2 rats by day 2 compared with ETX-RA rats ($P < 0.001$) and maintained a lower RV/LV+S ratio than in ETX-RA rats throughout the study period ($P < 0.001$ for each age). Both ETX-O2 and SA-O2 groups had RV/LV+S ratios comparable to SA-RA controls at each time point except at day 2 ($P < 0.05$, SA-O2 vs. SA-RA). There was no difference in the RV/LV+S ratios between ETX-O2 and SA-O2 group at each time point.

Percutaneous Oxygen Saturation

$SatO_2$ was decreased in ETX-RA newborn rats throughout 8 h of age compared with SA-RA controls (Fig. 8; $P < 0.05$). $SatO_2$ increased in ETX-RA rats to similar values as measured in SA-RA controls at day 1 and remained comparable to SA-RA controls from day 2 through day 14.

Western Blot Analysis

At birth, lung VEGF and VEGFR-2 protein expression was decreased in ETX-exposed rats compared with saline controls ($P < 0.01$ for VEGF; $P < 0.05$ for VEGFR-2; Fig. 9, A and B). At postnatal day 5, ETX-RA rats had lung VEGF and VEGFR-2
proteins comparable to SA-RA controls (Fig. 10, A and B). Lung VEGF protein in ETX-O2 rats was higher than ETX-RA rats and SA-O2 rats ($P < 0.05$, ETX-O2 vs. ETX-RA; $P < 0.01$ ETX-O2 vs. SA-O2; Fig. 10A). Lung VEGFR-2 protein in ETX-O2 rats was higher than the other three groups ($P < 0.05$ for each comparison; Fig. 10B). Lung eNOS protein content did not differ between the groups at birth or day 5.

Effects of Severe Neonatal Hyperoxia on Lung Structure After Intra-amniotic ETX

In a separate protocol, rat pups received intra-amniotic saline or ETX and were raised in more severe hyperoxia ($F_{O_2}$ ≥95% $O_2$) through 14 days of age. Figure 11 shows the comparison of the effects of 80% $O_2$ vs. ≥95% $O_2$ after intra-amniotic ETX on distal air space structure at day 14. Compared with ETX-RA and ETX-95% $O_2$ rats, ETX-80% $O_2$ rats had lower MLI and interstitial thickness. MLI in ETX-80% $O_2$ rats was comparable to SA-RA controls, whereas MLI in ETX-95% $O_2$ was higher than SA-RA controls ($P < 0.05$). Alveolarization was greater in ETX-95% $O_2$ rats than ETX-RA rats, but was less striking than in ETX-80% $O_2$ rats. In detailed microscopic examinations, scattered but more profound alveolar simplification was noted in four out of six ETX-95% $O_2$ rats compared with ETX-RA rats.

Fig. 10. Effects of intra-amniotic ETX and moderate postnatal hyperoxia on lung protein contents of VEGF, VEGFR-2, and eNOS at postnatal day 5. $P < 0.01$ vs. SA-RA. $P < 0.05$ ETX-O2 vs. ETX-RA.
shown in Fig. 11). SA-95% O₂ rats had fewer and larger alveoli and more interstitial thickening than SA-RA and SA-80% O₂ rats. There was no difference in lung morphometric analyses between SA-RA and SA-80% O₂ rats. When compared with SA-RA controls, SA-95% O₂ rats had increased MLI (P < 0.05), and SA-80% O₂ had MLI comparable to SA-RA. There was no difference in MLI between SA-80% O₂ and SA-95% O₂ groups. In room air, ETX-rats had higher MLI than saline controls (P < 0.001), but in 80% O₂ and in ≥95% O₂, there was no difference in MLI between ETX-rats and saline controls.

Figure 12 shows the comparison of the effects of 80% O₂ and ≥95% O₂ after intra-amniotic ETX on pulmonary vessel density at day 14. Compared with ETX-RA, pulmonary vessel density increased in the ETX-80% O₂ group (P < 0.001 vs. ETX-RA), but decreased in the ETX-95% O₂ group (P < 0.001 vs. ETX-RA). ETX-80% O₂ rats had similar pulmonary vessel density as measured in SA-RA controls, whereas ETX-95% O₂ rats had lower pulmonary vessel density than SA-RA controls (P < 0.05). Compared with SA-RA controls, both SA-80% O₂ and SA-95% O₂ groups had decreased pulmonary vessel density (P < 0.05 vs. SA-RA, respectively); there was no difference between SA-80% O₂ and SA-95% O₂ groups. In room air and in ≥95% O₂, ETX-rats had lower pulmonary vessel density than saline controls (P < 0.01, respectively), but in 80% O₂ there was no difference in pulmonary vessel density between ETX-rats and saline controls.

DISCUSSION

We found that exposure of fetal rats to a single dose of intra-amniotic ETX during the late canalicular stage of lung development caused sustained abnormalities of alveolar and vascular growth that persisted during infancy. The marked reduction in alveolarization and vascular growth led to altered lung structure and pulmonary hypertension that mimic features of human BPD. Antenatal ETX also caused right ventricular hypertrophy (RVH) and transient hypoxemia at birth and significant mortality in neonatal rats, suggesting that intrauterine inflammation can induce fetal lung vascular changes that lead to persistent pulmonary hypertension of the newborn (PPHN). Moreover, intra-amniotic ETX-induced pulmonary hypertension was sustained during infancy, which was associated with pulmonary vascular remodeling and decreased vessel density. These findings suggest that intrauterine inflammation may be sufficient to induce prolonged impairment of vascular growth and structure characteristic of BPD, and may contribute to the pathogenesis of neonatal pulmonary hypertension.

Rather than augmenting impairment of lung growth after antenatal ETX as we initially hypothesized, moderate postnatal hyperoxia actually accelerated alveolar and lung vascular growth, prevented lung vascular remodeling and pulmonary hypertension, and improved somatic growth in ETX-exposed infant rats. In contrast, severe postnatal hyperoxia further worsened lung vascular growth and generated a heterogeneous pattern of additional alveolar simplification after intra-amniotic ETX exposure, sug-
gesting dose-related effects of supplemental oxygen on lung structure after antenatal inflammation. In addition, lung VEGF and VEGFR-2 protein contents were decreased at birth in ETX-exposed rats, but were upregulated during moderate postnatal hyperoxia in infant rats after antenatal ETX. Previous research has shown that exogenous VEGF improved alveolar and lung vascular growth in rodent models of BPD (31, 32, 60). Whether these changes in lung VEGF signaling mediate the early adverse effects of antenatal ETX on the fetal and newborn rats or the accelerated lung growth response during moderate postnatal hyperoxia after antenatal ETX is uncertain.

Hyperoxia and oxidative stress have been shown to be major risk factors for the development of BPD in diverse animal studies. Previous studies have reported that severe neonatal hyperoxia worsens the impairment of neonatal lung growth after antenatal inflammation due to intra-amniotic ureaplasma infection (47) or E. coli ETX (9) in rodents. These studies are consistent with our findings that severe postnatal hyperoxia augments the impairment of lung structure in infant rats exposed to antenatal ETX. However, the present study further demonstrates that moderate neonatal hyperoxia actually restores alveolar and vascular growth in infant rats after intra-amniotic ETX. We found that neonatal oxygen treatment has dose-related effects on lung growth after antenatal ETX exposure, with moderate levels enhancing lung growth during infancy but severe hyperoxia causing additional impairment of lung structure after fetal ETX exposure. These differential effects contrast with the uniform effects of hyperoxia on saline-exposed rats, which demonstrated reduced lung vascular growth in response to both moderate and severe hyperoxia, in agreement with past studies of neonatal hyperoxia (55, 69). Interestingly, our laboratory findings parallel data from a recent multicenter clinical trial that demonstrated differential effects of targeted oxygen saturation on mortality and retinopathy in premature infants (7).

Mechanisms responsible for the dose-related effects of neonatal oxygen treatment on lung structure after antenatal inflammation are unclear. ETX induces synthesis of multiple proinflammatory cytokines in fetal sheep lung (27, 30) and activates NF-κB in neonatal mouse lung (2) and in fetal mouse lung explants in vitro (51). As downstream mediators of ETX, expression of NF-κB and proinflammatory cytokines is regulated by the redox status of lung cells (21, 34, 57). We speculate that different levels of postnatal hyperoxia after intra-amniotic ETX differentially modulate the balance of oxidative stress and inflammatory responses in the developing lung. In contrast, neonatal hyperoxia in the ETX-naïve lung simply causes inflammatory injury proportional to the intensity (56) and duration (65) of hyperoxia exposure. Alternatively, fetal ETX exposure might upregulate lung antioxidant defense mechanisms, as previously reported in a sheep model of chorioamnionitis (58). This mechanism may attenuate the inflammatory response to subsequent hyperoxia exposure, but it is unclear how increased antioxidant defenses would further enhance lung structure during hyperoxia. Similar findings have

Fig. 12. Comparison of 80% O₂ and ≥95% O₂ effects after intra-amniotic ETX on pulmonary vessel density. Lung histology was stained with vWF, and micrographs are representative for each group. There were 5–8 animals analyzed in each group. *P < 0.05 vs. SA-RA. HPF, high-powered field.
been reported regarding the interactions of neutrophilic inflammation and hyperoxia on lung structure in neonatal rats (72).

Mechanisms linking intra-amniotic ETX exposure with persistent abnormalities in infant rat lung structure and sustained pulmonary hypertension are unclear. Injury to the developing vasculature impairs angiogenesis in the neonatal lung, which can lead to persistent disruption in lung growth and sustained pulmonary hypertension during infancy, as shown in previous studies (24, 36, 59, 60). Clinical and experimental data suggest that antenatal inflammation alters fetal pulmonary vascular structure and function. For example, histological chorioamnionitis increases the risk for developing severe PPHN in term human infants (70), and intra-amniotic ETX causes pulmonary vascular remodeling (25) and elevates pulmonary arterial pressure (49) in preterm sheep, and these effects of intra-amniotic ETX are associated with decreased VEGF signaling and increased angiostatic chemokines in fetal sheep lung (25, 26). Whether decreased VEGF signaling or increased expression of angiostatic chemokines in the developing lung after intra-amniotic ETX contributes to sustained pulmonary hypertension needs further study. Whether decreased somatic growth with decreased alveolarization reflects poor nutritional intake, increased metabolism, or both in ETX-RA rats is uncertain. Interestingly, ETX-RA rats achieve a growth rate similar to SA-RA controls between days 7 and 14, yet these rats fail to restore alveolarization.

Studies on the interactive effects of moderate hyperoxia with ETX on lung injury in adult rodents have been inconsistent (1, 54). Unlike a previous study showing that breathing 60% O₂ significantly decreases lung neutrophil accumulation after ETX treatment of adult rats (54), Aggarwal et al. (1) recently showed that moderate hyperoxia (60% oxygen) worsens ETX-induced lung injury in adult mice, which was mediated by increased neutrophilic inflammation. These latter findings may reflect developmental differences in the response to injury; however, in contrast with this previous study in which exposure to ETX and hyperoxia were concurrent (1), ETX exposure preceded hyperoxia in our model.

Mechanisms through which moderate postnatal hyperoxia restores neonatal lung growth after intra-amniotic ETX exposure are unknown. Past studies have shown a potential role of NF-κB activation in protecting neonatal lung after ETX (2) and during hyperoxia (71). NF-κB, which can be activated by systemic ETX (2) and hyperoxia (71) in neonatal mouse lung, protects against ETX-induced lung inflammation in neonatal mice (2). Interestingly, disruption of NF-κB in the neonatal mouse increases lung inflammation in hyperoxia (71). Whether altered NF-κB activity after intra-amniotic ETX contribute to the effects of hyperoxia on restoring neonatal lung growth after antenatal inflammation requires further study.

Our findings differ from a previous study that showed modest abnormalities of lung structure in infant rats after exposure to much lower doses of intra-amniotic E.coli ETX during the late canalicular stage (9). Differences between these studies suggest that the intensity of intrauterine inflammation might determine its net impact on disrupting postnatal lung growth. This parallels clinical data that the degree of histological chorioamnionitis is directly associated with the severity of BPD in preterm infants (64).

Antenatal inflammation is also associated with an increased risk for retinopathy of prematurity in preterm infants (13, 42).

Retinal structure was not examined in the present study; the separate and combined effects of antenatal ETX and postnatal hyperoxia on retinal development were not studied in this model.

We conclude that exposure to intra-amniotic ETX induces fetal pulmonary hypertension, downregulates lung VEGF signaling in the newborn, and causes persistent impairment of lung growth with sustained pulmonary hypertension in infant rats. Moreover, moderate postnatal hyperoxia accelerates lung growth and attenuates pulmonary hypertension in infant rats exposed to antenatal ETX, whereas severe hyperoxia further disrupts lung vascular growth after antenatal ETX. We speculate that downregulation of lung VEGF signaling before birth might contribute to impaired lung growth in infant rats exposed to intra-amniotic ETX and that augmented lung VEGF signaling might contribute to restored lung growth in moderate hyperoxia after antenatal ETX exposure. We suggest that insights into mechanisms through which supplemental oxygen enhances lung growth after chorioamnionitis may improve long-term outcomes in prematurely born infants.

GRANTS

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the author(s).

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