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Sex-specific differences in neonatal hyperoxic lung injury

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Lingappan K, Jiang W, Wang L, Moorthy B. Sex-specific differences in neonatal hyperoxic lung injury. Am J Physiol Lung Cell Mol Physiol 311: L481–L493, 2016. First published June 24, 2016; doi:10.1152/ajplung.00047.2016.—Male sex is considered an independent predictor for the development of bronchopulmonary dysplasia (BPD) after adjusting for other confounders. BPD is characterized by an arrest in lung development with marked impairment of alveolar septation and vascular development. The reasons underlying sexually dimorphic outcomes in premature neonates are not known. In this investigation, we tested the hypothesis that male neonatal mice will be more susceptible to hyperoxic lung injury and will display larger arrest in lung development after postnatal day (PND) 1–5 and euthanized on PND 7 and 21. Extent of alveolarization, pulmonary vascularization, inflammation, and modulation of the NF-κB pathway were determined and compared with room air controls. Macrophage and neutrophil infiltration was significantly increased in hyperoxia-exposed animals but was increased to a larger extent in males compared with females. Lung morphometry showed a higher mean linear intercept (MLI) and a lower radial alveolar count (RAC) and therefore greater arrest in lung development in male mice. This was accompanied by a significant decrease in the expression of markers of angiogenesis (PECAM1 and VEGFR2) in males after hyperoxia exposure compared with females. Interestingly, female mice showed increased activation of the NF-κB pathway in the lungs when compared with males. These results support the hypothesis that sex plays a crucial role in hyperoxia-mediated lung injury in this model. Elucidation of the sex-specific molecular mechanisms may aid in the development of novel individualized therapies to prevent/treat BPD.

BRONCHOPULMONARY DYSPLASIA (BPD) is the leading cause of morbidity affecting premature babies with an incidence as high as 52% in extremely low birth weight (ELBW) (<1,000 g) neonates (50). It is well known that neonatal outcomes for males are worse than females for many diseases, including BPD. Male premature neonates have higher neonatal and infant mortality rates than females (16, 67). The incidence of BPD is lower among very low birth weight girls after adjusting for other confounders. Male sex is considered an independent predictor for the development of BPD (4, 16, 39, 56, 72, 78). The lung function in boys both in the neonatal period and at 1 yr of age was noted to be worse when compared with girls (68, 70). Despite the well-established sex-specific differences in the incidence of BPD and impaired lung function in males, the molecular mechanism(s) behind these are not completely understood.

BPD has long-term consequences such as chronic pulmonary morbidity, increased rehospitalization rates, development of pulmonary hypertension, and delayed neurodevelopment (1, 65). New longitudinal data demonstrate that survivors of BPD have longstanding deficits in lung function and may be at risk for the development of additional lung diseases as adults (7, 21). Current neonatal care is mainly supportive with few effective therapies that prevent or treat established BPD. The etiology of this disease is multifactorial, and exposure to high concentrations of oxygen (hyperoxia) postnatally contributes to its development via generation of reactive oxygen species (62).

In animal models, the effect of sex and sex hormones on lung physiology and disease has been studied. Hormonal, physiological, and developmental differences between males and females could lead to these sex-specific differences. Epidemiological data point to the effect of sex in the incidence, susceptibility, and severity of many lung diseases from the neonatal (respiratory distress syndrome, BPD) to the adult period (asthma, lung cancer, interstitial lung disease) (10). The modulation by sex hormones may contribute to the disease pathogenesis or serve as protective factors, depending on the disease involved.

Sex also has an effect on prenatal lung development. Fetal lung development, in particular surfactant synthesis, exhibits a sexual dimorphism (54, 63) due to the deleterious effects of androgens in the developing male fetus. Androgens also increase lung injury under hyperoxic conditions (51). However, mammalian cells also differ intrinsically based on sex and respond differently to stressors irrespective of the past or current concentrations of sex hormones (58). Even though hormones could be responsible for the differences in the outcomes, it is important to elucidate the underlying molecular mechanisms. The recently released NIH policy statement underlines the importance of sex-specific research in preclinical animal and in vitro studies (12). A focused investigation of the effect of sex/gender on neonatal hyperoxic lung injury and the underlying mechanisms has not been attempted. The objective of this research was to determine sex-specific differences in hyperoxic lung injury and the possible underlying mechanisms in wild-type (C57BL/6) male and female neonatal mice. We hypothesized that male wild-type (WT) neonatal mice will be more susceptible than females to hyperoxic lung injury and will display larger arrest in lung development after postnatal hyperoxia exposure.

METHODS

Animals. All animal experiments were performed under an approved protocol by the IACUC at the Baylor College of Medicine.
Timed pregnant C57BL/6j WT mice were obtained from Charles River Laboratories (Wilmington). The sex in neonatal mouse pups was determined by both the anogenital distance and pigmentation in the anogenital region method. In neonatal male mice, a pigmented spot on the scrotum is visible to the naked eye from postnatal day (PND) 1, whereas female pups lack visible pigmentation in the anogenital region. Since we used pigmented mice, this gave us 100% success rate of sex identification in our pups (75). On PND7 we reconfirmed the sex of the mice with PCR analysis for the Sry gene in genomic DNA obtained from mouse-tail clips before proceeding with any analysis (Fig. 1). The primer pairs used were as follows: Sry, forward 5′-TCATGAGACTGCCAACCACAG-3′ and reverse 5′-CATGACCACCACCCACCAACCCAA-3′; and myogenin, forward 5′-TTACGTCCATCGTGACAGC-3′ and reverse 5′-TGGGCTGGGTGTAGGTCTTA-3′.

Mouse model of BPD. An arrest of alveolarization was induced in mouse pups by exposure to hyperoxia (95% O2), as described previously (28, 40). Mouse pups from multiple litters were pooled before being randomly and equally redistributed to two groups, one group exposed to normoxia (21% O2) and the other group exposed to hyperoxia (95% O2), within 12 h of birth for 5 days. Animals at this stage of development were chosen because neonatal mice are at the saccular stage of lung development during this period, which is equivalent to 26–36 wk in human neonates. The litter size was limited to six pups to control for the effects of litter size on nutrition and growth. This model has previously been described and carefully characterized, where a pronounced arrest of lung development is seen in response to hyperoxia exposure. The dams were rotated between air- and hyperoxia-exposed litters every 24 h to prevent oxygen toxicity in the dams and to eliminate maternal effects between the groups. Oxygen exposure was conducted in Plexiglas chambers (55 × 40 × 50 cm), into which O2 was delivered through an oxygen blender (Thermo Scientific, Wilmington, DE). Sample Quality checks were performed on all samples to ensure RNA integrity.

Lung histology and morphometry. Both hyperoxia- and room air-exposed animals were anesthetized (100 mg/kg ip pentobarbital sodium), tracheas were cannulated, and lungs were fixed with 4% paraformaldehyde by instilling endotracheally at 25 cmH2O pressure for 15 min. The trachea was tied off and the lungs were removed and further fixed overnight at 4°C followed by dehydration in graded alcohol and embedded in paraffin. Sections (5 μm) were prepared and stained with hematoxylin-eosin and Masson’s trichrome stain. Alveolar development was evaluated at PND21 (n = 5/group) by radial alveolar counts (RAC) (14) and mean linear intercept (MLI) (47) as described before (52). Fifteen randomly chosen areas were photographed with a 10X objective of a microscope. Fields containing large airways and vessels were not included. Analysis of each section will be carried out in a blinded fashion.

Analysis of inflammation. Macrophage and neutrophil infiltration in the lung sections was quantified using F4/80 antibody for macrophages (1:500 dilution, Bio-Rad Laboratories; catalog no. MCA497GA) and rat anti-mouse Ly-6B.2 monoclonal antibody for neutrophils (1:500 dilution, Bio-Rad Laboratories; catalog no. MCA771GA). Twenty random nonoverlapping high-power fields were analyzed and numbers of cells were counted and the average number of cells per high-power field was calculated.

Lung RNA extraction and real-time qPCR analysis. Total RNA from lung samples in mice exposed to room air or hyperoxia was isolated on PND7 using the miRNeasy kit as per the manufacturer’s standard protocols (Qiagen, Valencia, CA). Following total RNA isolation, sample concentration was assayed using a Nanodrop-8000 (Thermo Scientific, Wilmington, DE). Sample Quality checks were performed on all samples to ensure RNA integrity.

Table 1. List of genes for qPCR analysis

<table>
<thead>
<tr>
<th>Gene Symbol</th>
<th>Assay ID</th>
</tr>
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<tbody>
<tr>
<td>Cyplal</td>
<td>Mm00487217_m1</td>
</tr>
<tr>
<td>IL-6</td>
<td>Mm00446190_m1</td>
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<td>TNF-α</td>
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<td>IL-1B</td>
<td>Mm00434228_m1</td>
</tr>
<tr>
<td>18s</td>
<td>Mm03928990_g1</td>
</tr>
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Fig. 1. PCR analysis of genomic DNA from mouse tails, showing Sry and control (myogenin) bands. M stands for molecular marker. A representative PCR analysis shows expression of Sry, a sex-determining region Y gene, in genomic DNA derived from neonatal male but not in female mice.

Fig. 2. A: body weights [postnatal day (PND) 21] in male and female neonatal mice exposed to hyperoxia (95% FiO2, PND 1–5). In the room air group: n = 12 male, n = 15 female; in the hyperoxia group: n = 7 male, n = 17 female mice. B: lung weight/Body weight ratios (mg/g) in male and female neonatal mice exposed to hyperoxia (95% FiO2, PND 1–5) on PND6 immediately after hyperoxia exposure (n = 4 animals/group). Values are means ± SE. Significant differences room air- and hyperoxia-exposed animals ##P < 0.01.
done using the NanoDrop spectrophotometer. RNA (50 ng), isolated as above, was subjected to one-step real-time quantitative TaqMan RT-PCR using 7900HT Fast Real-Time PCR System (Applied Biosystems, Foster City, CA). Gene-specific primers purchased from Life Science Technologies (Table 1) in the presence of TaqMan reverse transcription reagents and RT reaction mix (Applied Biosystems, Foster City, CA) were used to reverse transcribe RNA, and TaqMan Gene Expression probes and TaqMan Universal PCR Master Mix (Applied Biosystems, Foster City, CA), were used for PCR amplification. 18S was used as the reference gene.

**Pulmonary vascular development.** Pulmonary vessel density was determined based on immunofluorescence staining for vWF (1:4,000 dilution, Abcam; catalog no. ab6994), which is an endothelial specific marker. vWF-stained vessels with external diameter <100 μm per high-power field, 10 counts from 10 random nonoverlapping fields (×200 magnification), were performed for each animal (n = 6/group). The fields containing large airways or vessels were avoided.

**Western blot.** For protein samples, the lungs were excised and immediately frozen in liquid nitrogen until further use. The snap-frozen lung samples were weighed and added to 1 ml of lysis buffer (PBS + cocktail of protease inhibitor). The tissues were ground and the samples were incubated on a rocker at 4°C for 15 min followed by centrifugation at 10,000 g for 15 min. The supernatants were removed and protein concentrations were determined using the BCA (bicinchoninic acid) method. Lung whole protein (20 μg of protein) was prepared and subjected to SDS polyacrylamide gel electrophoresis in 10% acrylamide gels. The separated proteins on the gels were transferred to polyvinylidene difluoride membranes, followed by Western

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**Fig. 3.** Lung architecture (PND 21) in male and female neonatal mice (n = 6/group) exposed to hyperoxia (95% FiO2, PND 1–5). Representative hematoxylin and eosin stained sections form male and female neonatal mice exposed to room air or hyperoxia at 10× (A) and 20× (B) magnification.
After the membranes were blocked in 5% nonfat dry milk, they were incubated overnight with primary antibodies for goat anti-platelet endothelial cell adhesion molecule (PECAM/CD31; Abcam; catalog no. ab28364) 1:500 dilution; VEGFR2/Flk-1 1:500 dilution, NF-κB p65 (1:1,000 dilution, Cell Signaling, catalog no. 8242), phospho NF-κB p65 (Ser 536) (1:1,000 dilution, Cell Signaling, catalog no. 3033), p-IκB-α (1:1,000 dilution, Santa Cruz Biotechnology, catalog no. sc-8404), IKK-α/β (1:1,000 dilution, Santa Cruz Biotechnology, catalog no. sc-7607), or beta-actin (1:4,000 dilution, Santa Cruz, catalog no. sc-47778). The membranes were washed and incubated with the appropriate secondary antibodies. Beta-actin was used as the loading control. This was followed by electrochemical detection of bands. Band intensities were quantified using Image Studio Lite Software.

Immunofluorescence staining for phospho NF-κB p65. Phospho NF-κB p65 expression in the lung sections was quantified using phospho NF-κB p65 (1:500 dilution, Santa Cruz Biotechnology; catalog no. sc-33020).

Statistical analysis. GraphPad version 6 was used for the analysis of our data. Data are expressed as means ± SE. Sample size estimates were based on preliminary results of hyperoxia-induced alveolar simplification in WT neonatal mice, and it was estimated that 5 animals per group would provide 80% power at a significance level of 0.05 to find a desired significant mean difference of 5 μm in mean linear intercept (MLI) between the two groups (male and female). Data were analyzed by two-way ANOVA to test for the independent effects of sex and hyperoxia and to look for any interaction. Multiple-comparison testing (Bonferroni) was performed if statistical significance (P < 0.05) was noted by ANOVA.

RESULTS

Survival, body weight, and lung weight/body weight ratios of male and female neonatal mice exposed to hyperoxia. Male mice had higher mortality (9/26 = 34%) compared with female neonatal mice (3/29 = 10.3%) upon exposure to hyperoxia (P < 0.05). We recorded the body weights of the animals on PND21. Hyperoxia led to a decrease in the body weight in both male and female mice compared with room air controls; however, this was significant in male (P < 0.01) but not female mice. These results are shown in Fig. 2A. As a measure of pulmonary edema, we measured lung weight/body weight (LW/BW) ratio on PND6 immediately after hyperoxia exposure (45). Hyperoxia increased LW/BW ratios (Fig. 2B) in both male...
male and female neonatal mice with no sex-specific differences.

Male mice had increased alveolar simplification following postnatal hyperoxia exposure compared with females. Male and female newborn mice were exposed to room air or 95% O2 within 12 h of birth for 5 days (PND1-5) as described in METHODS. They were allowed to recover in room air until PND21. Lung morphometry was assessed to quantitate the effect of postnatal hyperoxia exposure in this model on lung development. Representative lung fields from room air- and hyperoxia-exposed animals are shown in Fig. 2 at 10× (Fig. 3A) and 20× magnification (Fig. 3B). Alveolar size was quantified using mean linear intercept (MLI). The MLI was not different between male and female mice kept in room air on PND 21. The MLI (Fig. 4A) was significantly increased in hyperoxia-exposed mice (P < 0.001), but this was higher in male mice compared with female mice (P < 0.001). We also measured radial alveolar counts (RAC) as a measure of postnatal alveolarization and to assess the effects of postnatal hyperoxia exposure. The RAC (Fig. 4B) was adversely impacted by hyperoxia (P < 0.001) but was decreased to a larger extent (P < 0.01) in male mice compared with similarly exposed female neonatal mice. Male mice showed a larger arrest in alveolarization compared with female neonatal mice following postnatal hyperoxia exposure. To assess lung fibrosis, lung sections were stained with Masson’s trichrome stain and representative sections are shown in Fig. 5. No increased collagen deposits were observed in hyperoxia-exposed animals in this model.

Pulmonary macrophage and neutrophil infiltration is higher in male mice following postnatal hyperoxia exposure compared with females. To assess sex-specific differences in lung inflammation in our model, we quantified macrophage infiltration on PND21 in the lungs by immunohistochemistry as described in METHODS. The representative lung sections are shown in Fig. 6A and the quantification in Fig. 6B. Macrophage infiltration was significantly increased in the lungs of hyperoxia-exposed mice (P < 0.001) compared with room air controls. This was higher in male mice (P < 0.001) compared with female mice. Hyperoxia increased neutrophil in lung

![Fig. 6. Immunohistochemistry and quantitation of pulmonary macrophage recruitment. A: representative immunostained images for lung macrophages. Hyperoxia-induced macrophage recruitment was determined by immunohistochemistry with anti-macrophage antibodies in male and female mice (n = 5 male, room air; n = 6 male, hyperoxia; n = 5 female, room air; n = 10 female, hyperoxia) in room air or hyperoxia (95% FiO2, PND 1–5). Arrows point to brown-staining macrophages. B: quantitative analyses showing number of macrophages per high-power field. Representative quantitative analysis of the hyperoxia effects on macrophage recruitment in lungs of male vs. female mice. Values are means ± SE from 5–10 individual animals. Significant differences between room air and hyperoxia ###P < 0.001. Significant differences between male and female mice: ***P < 0.001.](http://ajplung.physiology.org/ by 10.220.33.6 on October 29, 2017)
parenchyma in exposed neonatal mice on PND21 compared with room air controls. The representative lung sections are shown in Fig. 7A and the quantification in Fig. 7B. This increase was larger in male compared with female neonatal mice.

Differential sex-specific cytokine response in the lung in response to neonatal hyperoxia exposure. To assess differences in the inflammatory response in the lung following neonatal hyperoxia exposure we quantified mRNA expression of various inflammatory genes. These results are shown in Fig. 8. There was significant upregulation of IL-1β and TNF-α expression in male lungs but not in female lungs. A trend toward higher expression of CxCl1 and CxCl2 was also noted in males; however, this was not statistically significant. IL-6 mRNA was upregulated in both male and female hyperoxia-exposed animals. CCl2 mRNA was upregulated in female but not in male neonatal mice on PND7 following hyperoxia exposure.

Arrest in angiogenesis is greater in male mice following postnatal hyperoxia exposure compared with female mice. Pulmonary angiogenesis is critical for alveolarization, and arrest in vascular development adversely affects lung development (33, 69). Hyperoxia exposure during lung development also causes an arrest in vascular development in addition to alveolarization. To assess differences in angiogenesis among male and female neonatal mice, we quantified vessel number using anti-vWF antibodies as described in Methods. These results are shown in Fig. 9, A and B. Hyperoxia significantly decreased vessel development in the lung in both male and female mice (P < 0.01) but the degree of impairment was larger in male neonatal mice exposed to hyperoxia (P < 0.001). We also quantified the level of PECAM1/CD31, which is an endothelial specific marker in whole lung protein using Western blot. The results are shown in Fig. 10A. Hyperoxia significantly decreased (P < 0.05) the expression of PECAM1 in the lungs of male mice.

![Fig. 7. Immunohistochemistry and quantitation of pulmonary neutrophil recruitment. A: representative immunostained images for lung neutrophils. Hyperoxia-induced neutrophil recruitment was determined by immunohistochemistry with anti-neutrophil antibodies in male and female mice (n = 5/group) in room air or hyperoxia (95% FiO2, PND 1–5). Arrows point to brown-staining neutrophils. B: quantitative analyses showing number of neutrophils per high-power field. Representative quantitative analysis of the hyperoxia effects on neutrophil recruitment in lungs of male vs. female mice. Values are means ± SE from 5 individual animals. Significant differences between room air and hyperoxia #P < 0.05, ###P < 0.001.](http://ajplung.physiology.org/)

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growth of male neonatal mice compared with similarly exposed female neonatal mice ($P < 0.05$). We also measured the expression of VEGFR2/Flk-1 levels (Fig. 10B), which is also an important driver of postnatal angiogenesis in the lung (13, 29). The expression of Flk-1 protein was decreased in hyperoxia-exposed male neonatal mice ($P < 0.05$) but not in female neonatal mice compared with room air controls.

**NF-κB pathway is modulated in a sex-specific manner in the developing lung following postnatal hyperoxia exposure.** Sustained activation of the NF-κB pathway has been shown to be important in regulating postnatal angiogenesis and alveolarization (48). We hypothesized that there would be sex-specific differences in modulation of this pathway. We measured the expression of NF-κB p65 (Fig. 11A) and phosphorylated NF-κB [phospho-NF-κB p65 (Ser536)] (Fig. 11B) expression in male and female neonatal mice exposed to hyperoxia. There was decreased NF-κB p65 expression in female neonatal mice exposed to hyperoxia compared with room air controls but the expression of phospho-NF-κB p65 (Ser536) was increased in the lungs of female mice exposed to hyperoxia compared with males.

**Differential sex-specific expression of pulmonary Cyp1a1 mRNA in neonatal lungs.** We have previously reported on the protective effects of cytochrome P450 (CYP)1A1 against hyperoxic lung injury in adult and neonatal mice (17, 43, 44). To assess sex-specific differences in the Cyp1a1 mRNA in the lungs at room air and following neonatal hyperoxia exposure we quantified Cyp1a1 gene expression using RT-PCR. These results are shown in Fig. 12. Female neonatal mice (PND7) had significantly higher Cyp1a1 mRNA expression compared with males. The expression was decreased in the lungs of hyperoxia-exposed animals.

**DISCUSSION**

Sex is an important biological variable, and there is renewed impetus on research on sex-specific mechanisms of lung diseases as a step toward novel therapeutic approaches and individualized medicine. The male disadvantage in neonatology has been known for many years both in terms of mortality and major morbidities such as BPD and intracranial hemorrhage (6,
Even in the post-surfactant era, Binet et al. (4) reported that male extremely premature neonates (born between 24 and 26 wk of gestation) displayed a significantly increased risk of respiratory complications. The underlying molecular mechanisms are not well known and need to be studied. Our study shows that sex-specific differences exist in our model of postnatal hyperoxic lung injury in C57BL/6J mice. We show that neonatal male mice are more susceptible

Fig. 9. Immunohistochemistry and quantitation of pulmonary vessels. A: representative immunostained images for vWF (endothelial-cell specific marker). Effect of hyperoxia on pulmonary vascular development was determined by immunohistochemistry with anti-vWF antibodies in male and female mice (n = 6 mice per group) in room air or hyperoxia (95% FiO2, PND 1–5). Arrows point to brown-staining vessels; B: quantitative analyses showing number of vessels per high-power field in lungs of male vs. female mice. Values are means ± SE from 6 individual animals. Significant differences between room air and hyperoxia ##P < 0.01 and ###P < 0.001. Significant differences between male and female mice: ***P < 0.001.

Fig. 10. Effect of hyperoxia on PECAM1 and Flk-1 protein expression in neonatal mouse lung. Representative Western immunoblots (A) and densitometric analysis of pulmonary PECAM1 (B) and Flk-1 (C) isolated from WT male and female neonatal mice (PND 7) exposed to room air or hyperoxia (95% FiO2, PND 1–5). Under each sample lane is the corresponding beta-actin blot to account for protein loading. Values are means ± SE from 3 individual animals. Significant differences between room air and hyperoxia within each sex: #P < 0.05.
and have a higher mortality and arrest in alveolarization and pulmonary angiogenesis compared with similarly exposed female mice. They also exhibit more inflammation in the lungs. Furthermore, we also highlight the differential modulation of the NF-κB pathway in our model in male and female mice. We did not find increases in lung fibrosis in our study with the current model. This could be because of the brief duration of hyperoxia exposure in our study, and similar results have been reported in other studies (23).

The role of sex has been studied in many pulmonary diseases such as asthma, COPD, pulmonary fibrosis, and pulmonary hypertension. Casimir et al. (11) reported worse prognosis in males in acute diseases such as hyaline membrane disease, sepsis, and meconium aspiration syndrome and in females with chronic diseases such as asthma and COPD. In adult mice, we have previously shown sex-specific changes in acute hyperoxic lung injury (41). Adult (6–8 wk old) male mice showed more lung injury, apoptosis, and inflammation compared with similarly exposed female mice. We also showed that the cytochrome P4501A (CYP1A) enzymes showed sex-specific differences in this model (43). We further highlighted the changes in the pulmonary transcriptome in the acute lung injury model in adult mice and elucidated the sex-specific changes in gene expression in the lung (42).

Hyperoxia contributes to the development of BPD in the premature neonate probably by increasing oxidative stress in the developing lung (3). Hyperoxia leads to the production of reactive oxygen species (ROS) and these molecules lead to lung injury via oxidation of cellular macromolecules including DNA, protein, and lipid (25). We chose to limit hyperoxia exposure to PND1-5 in our model as neonatal mice are at the saccular stage of lung development during this period, which is equivalent to 26–36 wk gestation in human neonates (2, 77). Yee et al. (77) showed persistent effects of hyperoxia exposure during the saccular stage (PND1-4) at 8 wk on lung structure and function. This simulates the clinical course of most premature neonatal hyperoxic lung injury.

**Fig. 11.** Sex-specific differences in the effect of hyperoxia on NF-κB pathway in neonatal mouse lung. Representative Western immunoblots and densitometric analysis of pulmonary NF-κB p65 (A), phosphorylated NF-κB [phospho-NF-κB p65 (Ser536)] (B), p-IκB-α (C), and IKK-α/β (D) isolated from WT male and female neonatal mice (PND 7) exposed to room air or hyperoxia (95% FiO2, PND 1–5). Under each sample lane is the corresponding beta-actin blot to account for protein loading. Values are means ± SE from 3 individual animals. Significant differences between room air and hyperoxia within each sex: #P < 0.05 and ###P < 0.001. E: the representative immunostained images for phosphorylated NF-κB (phospho-NF-κB p65) in WT male and female neonatal mice (PND 21, n = 6/group) exposed to room air or hyperoxia (95% FiO2, PND 1–5). Arrows point to brown staining for the protein in bronchial epithelial and alveolar epithelial cells.
mature neonates who need respiratory support initially but are subsequently weaned off. We used 95% FiO2 in the present study, which does not simulate the clinical course of most human premature neonates in the NICU except for the sickest infants. Different effects may be seen with lower concentrations of inspired oxygen. Yee et al. (77) showed impaired lung alveolarization at 8 wk of age in a similar model with 60% FiO2 (PND1-4) but not with 40% FiO2 (77).

Some studies have looked into the possible mechanisms underlying better pulmonary outcomes in female preterm neonates. Better antioxidant defense mechanism in female neonates may contribute to this advantage (71, 73). Tondreau et al. (71) found transient lower expression of glutathione peroxidase 1 in male mice during the saccular stage (up to PND5) and reported a sexual dimorphism in murine lung enzymatic antioxidant defenses. Male mice were found to have lower lung superoxide dismutase (SOD) content and failed to upregulate SOD activity upon hyperoxia exposure (22). Vento et al. (73) reported less oxidative stress and increased antioxidant activity in human female preterm neonates. Sex-specific differences in adult lung architecture have been documented in mice exposed to postnatal hyperoxia (55). In this study alveolarization (as measured by MLI and RAC) was impaired in neonatal male mice compared with female mice.

Sex hormones, particularly androgens, negatively affect fetal lung development via a mechanism dependent on the presence of androgen receptors and are linked with the delay in the surfactant surge occurring at mid to late gestation in males, possibly leading to a higher incidence of RDS in premature male neonates (53). With respect to acute lung injury due to hyperoxia, Neri et al. (51) showed that castration prolonged tolerance of young (20 days old) male rats to pulmonary O2 toxicity. In other acute lung injury models, testosterone was found to increase (9) and estrogen to ameliorate inflammation and injury (66). After birth, both male and female newborns have similar plasma estrogen profiles, which are close to zero. Sex hormone differences at birth are mainly due to differences in testosterone levels (15). The postnatal testosterone surge is conserved in many mammalian species and has downstream physiological effects, which translate to sex differences (15). The developing lung both responds to and actively metabolizes androgens (63). Even though these findings could be explained by sex hormone-mediated effects, differences in sex-specific modulation of pathways activated by postnatal hyperoxia, which are hormone independent, could also be playing a part in these findings.

Inflammation secondary to recruitment of macrophages and neutrophils in the lungs has been shown to adversely affect lung alveolarization (20, 32, 36) and inflammatory cells (57) and levels of proinflammatory cytokines (such as IL-1β, IL-6, TNF-α) (35, 38) are increased in tracheal aspirates of human premature neonates who develop BPD. Male neonatal mice had higher pulmonary macrophage infiltration after postnatal hyperoxia exposure compared with females. The biological and clinical features associated with sexual dimorphism in inflammation have been reported. As a hematological parameter monocyte count was increased in males (9). There are several proteins linked with immunity that are encoded on the X chromosome. These include proteins related to toll-like receptor signaling pathway (Interleukin 1 receptor Y-associated kinase 1; IRAK 1) and NF-κB pathway (NF-κB essential modulator) (11). We also found increased neutrophil recruitment in the hyperoxia-exposed animals on PND21; the increase from room air controls was greater in male mice. Analysis of mRNA expression of various inflammatory genes on PND7 from male and female mice revealed several interesting sexually dimorphic differences. IL-1β and TNF-α were elevated in male mice. There was a trend toward higher expression of CxCl1 and CxCl2. IL-6 did not show any sex-specific differences in expression. Interestingly, CCI2 (MCP-1) was elevated to a greater extent in hyperoxia-exposed female mice. This has also been observed in other models of lung injury in adult mice where females had significantly elevated MCP-1 concentrations compared with males (6, 27). These sex-specific differences in the inflammatory response to postnatal hyperoxia exposure could in part explain the differential effects of postnatal hyperoxia on lung alveolarization in male and female mice.

Pulmonary angiogenesis is critical for alveolarization, and arrest in vascular development adversely affects lung development (33, 69). Exposure to high concentration of oxygen postnatally decreases vascular development in the lung (59). Hyperoxia decreases the expression of angiogenic factors such as VEGF and its receptors (29). PECAM1 is a proangiogenic endothelial cell surface molecule that promotes endothelial cell migration. Administration of anti-PECAM1 antibody disrupted alveolar septation, and PECAM1-null mice have impaired alveolarization (18). We show that following postnatal hyperoxia exposure, angiogenesis is impaired to a greater extent in male neonatal mice compared with females. The vessel number and the expression for PECAM1/CD31 and VEGFR2 were decreased in males. Keenaghan et al. (37) exposed neonatal rat pups of either sex to varying oxygen concentrations (10%–100% FiO2) for 2 h after birth and analyzed the pulmonary angiogenesis gene profiles. They reported that anti-angiogenesis genes including collagen type XVIII, and TIMP-3 were upregulated to a larger extent in males, and female pups were more resistant to the effects of hyperoxia (37).

Sustained activation of NF-κB and increased expression of downstream target genes have been shown to attenuate hyperoxia-induced mortality in adults and improve survival and preserve lung development in neonatal mice (49). Yang et al.
showed that enhanced NF-κB protects the neonatal lung from acute hyperoxic injury via inhibition of apoptosis. Franke et al. (24) showed that NF-κB decreased hyperoxia induced cell death in human pulmonary epithelial cells. In neonatal mice with LPS administration, NF-κB activation preserved alveolarization by inhibiting the anti-angiogenic cytokine macrophage inflammatory protein 2 (30). We showed enhanced activation of NF-κB in female neonatal mice compared with male mice in this study. NF-κB is usually localized to the cytoplasm as a heterodimer; the p50/p65(RELA) is the most abundant form. This complex is inhibited by IκB proteins and sequestered in the cytoplasm. Phosphorylation of IκB proteins by kinases (IKK-α/β) targets them for degradation, releasing the active NF-κB to enter the nucleus and activate gene expression. Phosphorylation of p65 subunit also plays a key role in the transcriptional activation after the nuclear translocation (34). Both IκB and p65 are substrates for the IKK complex that result in the activation of NF-κB (60). In human adults with acute respiratory distress syndrome, Fudala et al. (26) showed increased expression of p-p65 in lung tissue. In our study, female mice showed increased expression of phospho-NF-κB p65 (Ser536) in the lung following postnatal hyperoxia exposure. In male mice there was significant downregulation of IKK-β and p-IκB-α on PND7 following hyperoxia exposure. Immunohistochemistry for phospho-NF-κB p65 on PND 21 showed decreased expression in males following hyperoxia exposure. The larger decrease in angiogenesis in males exposed to hyperoxia may have been in part due to decreased NF-κB activation. Iosef et al. (31) reported that inhibiting NF-κB in the developing lung inhibited angiogenesis and that NF-κB was a direct regulator of VEGFR2 in the neonatal pulmonary vasculature.

The differences in lung morphometry and vascular development were obtained on PND21 when most of the alveolar development is completed in mice. However, the sex-specific effects on lung function and assessment of pulmonary hypertension remain to be determined in future studies. Moving forward genomewide changes in the transcriptome would provide further insight into the sex-specific modulation of pathways in this model. Even though we measured some angiogenic markers and factors others like VEGF, VEGFR1 and Angiopoietin 1/2/Tie-1/2 could potentially have sex-specific effects and need to be explored.

We have previously reported on the protective effects of (CYP)1A against hyperoxic lung injury in adult and neonatal mice (17, 43, 44) by decreasing oxidative stress. At baseline on PND7 female mice show increased expression of Cyp1a1 mRNA in the lungs, and levels decrease in both male and female animals following hyperoxia exposure. The decrease in Cyp1a1 mRNA expression has also been reported in other studies (46, 61, 64).

In conclusion, we show that sex plays a crucial role in hyperoxia-mediated lung injury. Sex-specific differences in alveolarization, angiogenesis, and inflammation could explain the increased incidence of BPD in male premature neonates. These could be sex-hormone-dependent or -independent effects. Elucidation of the sex-specific molecular mechanisms may aid in the development of novel individualized therapies to prevent/treat BPD.
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33. Keenaghan M, Cai CL, Kumar D, Valencia GB, Rao M, Aranda JV, Beharry KD. Response of vascular endothelial growth factor and angio-


