Airway Hyperreactivity Is Delayed after Mild Neonatal Hyperoxic Exposure

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Key Words

Hyperoxia · Wheezing · Preterm infants · Airway hyperreactivity · Neonatal mouse

Abstract

Background: Wheezing disorders are prominent in former preterm infants beyond the neonatal period. **Objectives:** We used a neonatal mouse model to investigate the time course of airway hyperreactivity in response to mild (40% oxygen) or severe (70% oxygen) neonatal hyperoxia. **Methods:** After hyperoxic exposure during the first week of postnatal life, we measured changes in airway reactivity using the in vitro living lung slice preparation at the end of exposure [postnatal day 8 (P8)] and 2 weeks later (P21). This was accompanied by measures of smooth muscle actin, myosin light chain (MLC) and alveolar morphology. **Results:** Neither mild nor severe hyperoxia exposure affected airway reactivity to methacholine at P8 compared to normoxic controls. In contrast, airway reactivity was enhanced at P21 in mice exposed to mild (but not severe) hyperoxia, 2 weeks after exposure ended. This was associated with increased airway α -smooth muscle actin expression at P21 after 40% oxygen exposure without a significant increase in MLC. Alveolar morphology via radial alveolar counts was comparably diminished by both 40 and 70% oxygen at both P8 and P21. **Conclusions:** These data

demonstrate that early mild hyperoxia exposure causes a delayed augmentation of airway reactivity, suggesting a long-term alteration in the trajectory of airway smooth muscle development and consistent with resultant symptomatology.

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Introduction

Wheezing disorders and asthma remain a major source of longer-term morbidity in former preterm infants [1, 2]. While this is most severe in infants with a history of bronchopulmonary dysplasia (BPD), even late preterm infants appear at risk, many of whom may only have exposure to modest supplemental oxygen [3–5]. Interestingly, the increased airway reactivity exhibited by former preterm infants usually manifests beyond the neonatal period as exemplified by the limited benefit for bronchodilator therapy in the neonatal intensive care unit [6]. Therefore, there is a need for animal models to characterize the developmental trajectory of airway function after neonatal lung injury.

Neonatal rodents have served as models to study respiratory mechanics after neonatal hyperoxia exposure and lung mechanics or airway hyperreactivity have been documented in vivo from 14 and 21 days of life [7–12]. Com-

parative data on lung development indicate that alveolar formation in rodents is largely complete by day 14, which approximates the lung of a 2-year-old infant [13, 14]. Unfortunately, the small size of neonatal mice precludes reliable *in vivo* measurement of airway reactivity prior to approximately day 14. However, the living lung slice model provides a novel technique to quantify changes in airway caliber in response to a bronchoconstrictor challenge under *in vitro* conditions [15]. This method allows airway contractility to be assessed with bronchoalveolar attachments intact as would occur *in vivo*, and we have adapted this technique to study lung slices in 1-week-old mice.

In this study we exposed mouse pups to mild (40% oxygen) or severe (70% oxygen) hyperoxia for the first 7 postnatal days and used living lung slices at postnatal (P) days 8 and 21 to test the following two hypotheses: (1) onset of airway hyperreactivity is delayed after neonatal hyperoxic exposure, and (2) mild hyperoxic exposure causes a greater increase in airway reactivity than severe hyperoxia as suggested by our prior *in vivo* studies employing lung mechanics. We also sought to correlate our physiologic data with changes in airway smooth muscle and lung morphology.

Methods

All procedures were carried out in accordance with the National Institutes of Health guidelines for laboratory animals and approved by the Animal Care and Use Committee at Case Western Reserve University. Time-pregnant mice (C57BL/6J) gave birth in our animal facility and together with the dam were assigned to receive 40 or 70% oxygen or room air for 7 consecutive days (control). Measurements of airway responses to methacholine using living lung slice preparation [15] were performed on 8 or 9 airways of comparable size per pup on either the day after exposure ended (P8) or at P21 (2-week recovery in room air). We studied only male mice to avoid an effect of sex on airway hyperresponsiveness [16].

For immunohistochemistry and morphology, the trachea was cannulated and the lungs were inflation fixed (25 cm H₂O) for 10 min with 10% neutral-buffered formalin as we have previously described [11]. The left lung was removed and postfixed for 2 days at 4°C and embedded in paraffin, and 5- μ m paraffin sections were processed. In those studies we analyzed 2–3 airways of comparable size (100–320 μ m in diameter) in each pup. For all experiments, group sizes comprised 5–7 pups.

Hyperoxia Exposure

Hyperoxic exposure consisted of the dam and her litter being placed inside a plexiglass chamber (30 L $\times$ 50 W $\times$ 28 H cm), through which gas flowed (3 l/min) at the desired concentration (room air, 40 or 70% oxygen). Oxygen concentrations were monitored and achieved using manual flow controllers as we have previously described [11]. Mice were exposed 24 h/day for 7 consecutive days starting the day after birth (P1). Dams were cycled be-

tween litters exposed to room air and hyperoxia every 24 h to minimize acute oxygen toxicity and to ensure that the normoxic and hyperoxic dams received the same exposure. At the end of the seventh day of exposure, the pups were removed from the chamber and then raised for a further 14 days in normoxia. Cages, water, and food were replaced every 3 days. Normoxic mice received room air for the same time period.

Hyperoxic exposure resulted in a decrease in body weight at P8 after 70% oxygen exposure in the pups who underwent physiologic study (room air: 5.05 ± 0.25 g; 40% oxygen: 4.56 ± 0.19 g; 70% oxygen: 3.91 ± 0.3 g; $p < 0.02$). At P21, body weights no longer differed [room air: 9.92 ± 1.05 g; 40% oxygen: 11.02 ± 0.36 g; 70% oxygen: 8.77 ± 0.67 g; n.s.].

Measurement of Airway Reactivity and in vitro Living Lung Slice Preparation

At P8 and P21, mice were sacrificed via anesthetic overdose, intraperitoneal injection of a ketamine (500 mg/kg)-xylazine (100 mg/kg) mix, to prepare the lungs for *in vitro* measurements of airway reactivity to methacholine. The trachea was cannulated and agarose gently injected to inflate the lungs (0.3–0.4 ml for P8 mice; 0.7–0.8 ml for P21 mice) until inflation volumes of 0.3–0.4 and 0.7–0.8 ml were achieved at P8 and P21, respectively. After the agarose was cooled and allowed to gel, the entire lung was removed, placed on a vibratome, sliced into 300- μ m sections, and immersed in DMEM solution for overnight incubation (5% CO₂; 37°C). The following day the lung slices were rinsed in Hanks' balanced salt solution (HBSS) and mounted in an *in vitro* recording chamber for live imaging of airway responses to methacholine challenge. Slices were obtained from the left lobe of each animal and sectioned transversely from lateral to medial.

For live imaging of individual airways, the slices were held covered with a thin lightweight sheet of mesh, which was also held in place with petroleum jelly. The recording chamber containing the slice was mounted on a microscope (Leica DMLFS) and perfused (7 ml/min) continuously with HBSS using a perfusion pump (MPII Harvard Instruments). The microscope mounted with a camera (Rolera Fast, Q Imaging, Canada) was used to identify individual airways under high magnification ($\times 5$). After an initial 3-min baseline period, the lung slice and chamber were perfused with increasing concentrations of methacholine and continuous infusion of HBSS at 7 ml/min was maintained both prior to and during methacholine application. The extent of constriction in response to methacholine doses of 0.25, 0.5, 1, 2, 4, and 8 μ M was determined from computer software (ImageJ) imaging of the luminal surface area at the end of a 2-min drug exposure. The agarose that had been perfused into the lungs to allow for cutting of the slice is often dislodged from the airway during the cutting procedure of overnight incubation. The presence of agarose in the airway was examined under a microscope before the methacholine challenge was performed, and those airways containing agarose were excluded from the study as this may have influenced airway caliber.

Airway Immunohistochemistry and Alveolar Morphometry

Immunohistochemistry for α -smooth muscle actin was performed as previously described [11], employing a monoclonal antibody against mouse anti- α -smooth muscle actin (1:400 dilution; Sigma-Aldrich). Immune complexes were captured with FITC-conjugated donkey anti-mouse secondary antibodies (1:500; Alexa

Fig. 1. Airway responses to methacholine challenge in the in vitro living lung slice preparation from P8 mice (**a**, **b**) that received 1 week of neonatal (P1–P7) hyperoxia (40 or 70% oxygen). Measurements were made on similar size airways in each of the 5 pups for each group (**c**). Airway responses to methacholine challenge were similar between treatment groups. Means $\pm$ SEM; 1 μm = 0.39 pixels.

Fig. 2. Airway responses to methacholine challenge in the in vitro living lung slice preparation in P21 mice (**a**, **b**) that received 1 week of neonatal (P1–P7) hyperoxia (40 or 70% oxygen). Measurements were made on similar size airways in each of the five pups for each treatment group (**c**). Note: there was an augmented airway constriction in 40% oxygen-exposed mice compared to control, whereas 70% exposure had no effect on airway reactivity. * Significant difference from control mice ($p < 0.05$). Means $\pm$ SEM; 1 μm = 0.39 pixels.

Fluor-488; Invitrogen). Individual airways were imaged (Rolera XR CCD camera Q Imaging) and the amount of smooth muscle actin area (μm^2) was analyzed with software (Image-Pro Plus 7.0; Media Cybernetics, Silver Spring, Md., USA). The green fluorescent areas of α -smooth muscle actin area were normalized for airway size using the basement membrane circumference.

Myosin light chain (MLC) immunohistochemistry employed rabbit anti-MLC polyclonal antibody specific for phosphorylated MLC (1:500; ab2480 Abcam, Cambridge, Mass., USA). The secondary antibody was biotinylated goat anti-rabbit (1:500; Jackson ImmunoResearch, West Grove, Pa., USA) followed by an ABC reagent, visualized by diaminobenzidine (Vectastain; Vector Laboratories, Burlingame, Calif., USA). Primary antibody was omitted for negative control. The MLC area was also normalized to the basement membrane perimeter.

Hematoxylin-eosin-stained sections were used to assess radial alveolar counts as we have previously described [11] by counting the number of alveolar septa transected by a perpendicular line drawn from the terminal bronchioles to the nearest connective tissue septum.

Statistics

Statistical comparison of airway reactivity to methacholine and body weight was performed between treatment groups using a two-way, repeated-measure ANOVA for each age group or between age groups where appropriate. Comparison of airway and lung morphology (area of actin and MLC, radial alveolar counts) in each age group was performed using a one-way ANOVA with a Student-Newman-Keuls post hoc test. Differences were considered significant at $p < 0.05$. All values are expressed as means $\pm$ 1 SEM.

Fig. 3. Comparison of airway responses to methacholine ($8 \mu\text{M}$) between P8 and P21 mice for each treatment group. Note: the magnitude of the response to methacholine (no differences between slopes, left panel) was similar between P8 and P21 control mice, whereas airway reactivity was significantly higher at P21 (steeper slope, middle panel) compared to P8 following 40% oxygen exposure. 70% oxygen exposure had similar effects on P8 and P21 airway reactivity (right panel). # Significant difference in the slope compared to P8 mice ($p < 0.05$). Means $\pm$ SEM; $1 \mu\text{m} = 0.39$ pixels.

Fig. 4. Representative contractile responses in airways from P21 mice previously exposed to room air (control) or 40 or 70% oxygen. Note: the greater decrease in lumen size in the 40% exposed tissue in response to $8 \mu\text{M}$ methacholine. Scale bar = $100 \mu\text{m}$.

Results

Airway Reactivity to Methacholine Challenge in 1- and 3-Week-Old Mice

Airway responses to methacholine challenge in P8 male mice are provided in figure 1. The slope of the airway response and maximal contraction to increasing doses of methacholine were not significantly different between treatment groups (fig. 1a, b). For consistency, airways of similar size were chosen as indicated by the comparable baseline airway lumen area (fig. 1c). These data suggest that a week of mild (40% oxygen) or severe (70% oxygen) neonatal hyperoxia exposure does not affect airway responses to methacholine challenge. In contrast, mild hyperoxia increased airway reactivity to methacholine at P21, 2 weeks after treatment ended, as indicated by the larger decrease in airway luminal area (fig. 2a, b) compared to control mice. However, airway responses to methacholine were similar between control mice and severe hyperoxia exposure.

Control mice raised in room air were equally reactive to methacholine ($8 \mu\text{M}$) at P8 compared to 3-week-old mice (fig. 3, left panel). However, 3-week-old mice exposed to mild hyperoxia exhibited an enhanced airway response at P21 than at 1 week, as indicated by the steeper slope of the response to maximum methacholine challenge (fig. 3, middle panel). In contrast, the slope of the response to methacholine was similar between 1- and 3-week-old mice exposed to severe hyperoxia. Representative contractile responses at P21 are shown in figure 4 after room air (control) or 40 or 70% oxygen exposure. These data indicate that mild neonatal hyperoxia does not affect airway reactivity at the time of ex-

Fig. 5. Immunohistochemical quantification of α -smooth muscle (α SM) actin in P8 and P21 mice following neonatal hyperoxia exposure. The α SM actin area expressed per airway (AW) basement membrane (BM) was similar between treatment groups at P8 (**a**), whereas at P21, it was increased by 40% but not 70% oxygen (**c**). Representative images are shown in **b** and **d**. * Significant difference from control and 70% exposed mice ($p < 0.05$). Means $\pm$ SEM. Ctrl = Control.

posure, but rather elicits a delayed effect that manifests approximately 2 weeks after treatment.

α -Smooth Muscle Actin, MLC, and Alveolar Morphology

At P8, there was no significant effect of oxygen exposure on α -smooth muscle actin expression (fig. 5a). At P21, oxygen exposure did have an overall effect on actin expression with a significant increase after 40% exposure when compared to both the room air control and 70%

oxygen groups (fig. 5c). Consistent with actin expression, MLC expression at P8 also did not differ between oxygen-exposed groups (fig. 6a). At P21, there was also no significant overall effect of treatment on MLC levels, although there was a trend for increased MLC expression after 40 versus 70% oxygen exposure (fig. 6b). Compared to room air controls, there was a significant and comparable decrease in radial alveolar counts after both 40 and 70% oxygen exposure which did not differ between P8 and P21 pups (fig. 6c, d).

Fig. 6. Immunohistochemical quantification of airway MLC and radial alveolar counts from P8 and P21 mice following neonatal hyperoxia exposure. The amount of MLC, normalized to basement membrane (BM) perimeter length, was similar between treatment groups at P8 and P21 (**a, b**), but there was a tendency for increased MLC following 40% oxygen exposure compared to 70% oxygen in P21 mice. Radial alveolar counts (**c, d**) were also reduced at both age groups exposed to either 40 or 70% oxygen compared to control mice (* both $p < 0.05$). Means $\pm$ SEM. Ctrl = Control.

Discussion

Although increased airway reactivity and resultant wheezing disorders are widely recognized clinical problems in former preterm infants, there is limited information on their natural history in the postneonatal period. This is attributable, in part, to the challenges of quantifying in vivo airway reactivity in both human infants and animal models during early postnatal development. Our study was motivated by the widespread clinical observation that airway hyperreactivity typically manifests beyond the neonatal period, especially in infants who do not have a history of BPD. We, therefore, chose to study the time course of airway hyperreactivity in a mouse pup model in response to mild versus severe hyperoxic expo-

sure. We hypothesized that the onset of airway hyperreactivity would be delayed after neonatal hyperoxic exposure, and that this might correlate with expression of airway smooth muscle contractile proteins.

We utilized living lung slice preparation which enables in vitro measurement of airway contractions in the presence of bronchoalveolar attachments and cell-matrix interactions [15, 17]. This technique provides a useful complement to in vivo measurements of airway resistance, especially in small animal models, such as neonatal mice, in which in vivo data of respiratory function with bronchoconstrictors have not been reported prior to 14 postnatal days [10, 11]. Unfortunately, the widely used flexi-Vent system for measuring respiratory function requires pups to be at least 7–8 g, making studies prior to approximately 14 days unreliable. It is very reassuring that our slice data at 3 weeks are comparable to our prior lung function studies [11].

Our major finding is that while neither mild nor severe hyperoxia increased airway contraction at P8, airway contractility significantly increased at P21, and only in mice exposed to mild hyperoxia. This occurred 2 weeks after cessation of exposure to 40% oxygen, suggesting a delayed effect of early neonatal hyperoxia on airway hyperreactivity. We have previously reported in 21-day-old mice that mild, but not severe, hyperoxia increased the response of respiratory system resistance to methacholine [11]. The current study employing lung slices confirms this finding and suggests that the living lung slice technique approximates the in vivo condition. We do recognize that an important limitation of the lung slice technique is the absence of an intact autonomic nervous system and accompanying circulation.

Mild hyperoxic exposure was associated with an increase in airway α -smooth muscle actin at P21. We also observed a trend toward increased MLC expression after 40 versus 70% oxygen exposure at P21. These findings are consistent with the observation of Hartman et al. [18] in human fetal airway smooth muscle cells that modest hyperoxia has a proliferative effect while severe hyperoxia causes apoptosis. We speculate that apoptosis induced by more severe hyperoxia [19] is the mechanism underlying the greater airway constrictor effect of 40 versus 70% oxygen exposure at P21. All preterm infants are at increased risk of wheezing disorders; however, this problem is enhanced in those with a history of BPD [1, 2]. While such infants may be exposed to higher or longer supplemental oxygen, this is typically accompanied by some form of positive pressure ventilation which was absent from our model of hyperoxic exposures.

We performed additional histologic studies to begin to identify a mechanism whereby neonatal hyperoxic exposure did not have an immediate effect on airway hyperactivity. Increased α -smooth muscle actin, as a measure of airway smooth muscle mass, only reached significance at P21 after prior 40% oxygen exposure. We also measured MLC content as a more specific contractile protein and observed no effect of oxygen at P8 and only a trend toward higher expression at P21 in 40 versus 70% exposure. Actin may be expressed by airway smooth muscle cells such as fibroblasts and myofibroblasts, which may contribute to constriction, and hence the stronger correlation between our physiologic findings and actin rather than myosin [20]. It is possible that the myosin data may have reached significance with larger group sizes. Future studies might focus on more specific contractile elements in airway smooth muscle and the relative roles of hypertrophy versus hyperplasia in our model.

Recent studies of neonatal lung injury have focused on the role of alveolar simplification in later respiratory morbidity of preterm infants with BPD [21], which might diminish tethering of small airway structures embedded in alveolar walls resulting in increased airway contractile responses [22]. However, radial alveolar counts were decreased to a comparable degree after both levels of hyperoxia at both P8 and P21, indicating it is unlikely that altered alveolar morphology is a major contributor to our findings of delayed airway hyperactivity after mild hyperoxic exposure. We did not quantify epithelial thickness in our slices; however, all our physiologic responses were based on initial lumen size which we attempted to match between airways. Therefore, any change in epithe-

lial thickness in response to hyperoxia should not have contributed to our findings. It should be noted that the lung slice technique does not provide a comparable measure to baseline lung resistance as would be obtained from *in vivo* pulmonary mechanisms. Three-week-old mouse pups were chosen to approximate the maturational age of human infants who exhibit a high incidence of wheezing following preterm birth. Future studies might address later ages as neonatal hyperoxia increases airway reactivity in mature mice [9] in a sex-dependent manner [16]. Although our prior *in vivo* study in developing mice did not demonstrate clear sex differences in airway reactivity [11], we chose to study only male pups to avoid confounding our current data with the previously described effect of sex on airway hyperresponsiveness in developing mice [16].

In conclusion, we have documented a delay in development of airway hyperactivity exhibited by mouse pups exposed to mild hyperoxia. Our data suggest this is associated with greater airway smooth muscle mass, although we cannot exclude other physiologic pathways contributing to the balance of airway contractile/relaxant mechanisms. This study provides new insight into airway-related symptomatology that preterm infants do not exhibit until the postneonatal period.

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