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Developmental Differences in Hyperoxia-Induced Oxidative Stress and Cellular Responses in the Murine Lung

Sara K. Berkelhamer^a, Gina A. Kim^a, Josiah E. Radder^b, Stephen Wedgwood^a, Lyubov Czech^a, Robin H. Steinhorn^a, and Paul T. Schumacker^a

Sara K. Berkelhamer: s-berkelhamer@northwestern.edu; Gina A. Kim: ginaakim9@gmail.com; Josiah E. Radder: jeradder@gmail.com; Stephen Wedgwood: s-wedgwood@northwestern.edu; Lyubov Czech: l-czech@northwestern.edu; Robin H. Steinhorn: r-steinhorn@northwestern.edu; Paul T. Schumacker: p-schumacker@northwestern.edu

^aDepartment of Pediatrics, 310 E. Superior St, Morton Building, Northwestern University, Chicago IL. 60611 USA

^bDepartment of Pulmonary and Critical Care Medicine, 240 E. Huron Ave, McGaw Mezzanine, Northwestern University, Chicago, IL. 60611 USA

Abstract

Exposure of newborn mice to high inspired oxygen elicits a distinct phenotype of compromised alveolar and vascular development, although lethality during long-term exposure is lower in newborns compared to adults. As the effects of hyperoxia are mediated by excessive reactive oxygen species (ROS) generation, we hypothesized that newborn mice may exhibit enhanced expression of antioxidant defenses or attenuated ROS generation compared with adults. We measured subcellular oxidant responses to acute hyperoxia in lung slices and alveolar epithelial cells at varying time points during postnatal murine lung development. Oxidant stress was assessed using roGFP, a ratiometric protein thiol redox sensor, targeted to the cytosol or the mitochondrial matrix. In contrast to newborn resistance to oxygen-induced mortality, cells of lung slices from younger mice demonstrated exaggerated mitochondrial matrix oxidant stress compared to adults, whereas oxidant stress responses in the cytosol were absent. Cell death in lung slices from newborn mice exposed to 48 hours of hyperoxia was also greater than for adults. Consistent with these findings, expression of antioxidant enzymes in newborn lungs was lower than in adults, and induction of antioxidant levels and activity during 24 hours of *in vivo* exposure was absent. However, expression of the reactive oxygen species generating enzyme, NADPH oxidase 1

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Corresponding Author: Sara K. Berkelhamer, MD, Department of Pediatrics, Northwestern University, 310 E. Superior St. Morton Bldg. 4-685, Chicago, IL 60611, Ph. 312 503 3971, Fax 312 503 1181, s-berkelhamer@northwestern.edu.

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(NOX1), was increased with hyperoxic exposure in the young but not adult lung. Collectively, these results suggest that the greater lethality in adult animals may be more likely attributed to processes such as inflammation than to differences antioxidant defenses. Therapies for neonatal and adult oxidative lung injury should therefore consider and address developmental differences in oxidative stress responses.

Keywords

Oxidative lung injury; lung development; subcellular response; mitochondrial oxidative stress; developmental differences; NADPH oxidase 1

INTRODUCTION

Supraphysiologic oxygen is used in the treatment of a wide range of pulmonary morbidities and in the resuscitation of newborn infants. However, exposure to high concentrations of oxygen may lead to hyperoxia-induced lung injury through the production of reactive oxygen species (ROS), including superoxide, hydrogen peroxide and the hydroxyl radical. Although ROS are by-products of normal oxygen metabolism, their increased generation in hyperoxia, especially when the cell's antioxidant defenses are limited, augments damage to proteins, lipids and DNA, leading to possible cell injury or altered cell proliferation. In addition, ROS are capable of promoting inflammation, resulting in the release of inflammatory mediators and secondary tissue damage (1–3). The end result of excess ROS generation is diffuse lung injury and, in the immature human and animal lung, the distinct phenotype of compromised alveolarization, thickened alveolar septa and vascular remodeling which parallels that seen clinically in bronchopulmonary dysplasia (BPD) (4–6).

Despite advances in neonatal care, BPD remains the most common complication of prematurity. While multiple factors contribute to the risk of disease, the strongest predictor is lower gestational age, placing the most premature infants at greatest risk (7–9). Animal models and studies in premature infants have established that expression and activity of multiple antioxidant enzymes (AOE) increase during the third trimester, presumably in preparation for higher oxygen exposure at birth (10–12). Infants born prematurely therefore have relative AOE deficiencies, upsetting the homeostasis of the cell. Disruption of this balance can enhance oxidative stress and lead to subsequent activation of apoptotic pathways or inhibition of normal lung growth and proliferation (13–15).

Maturation of antioxidants during lung development could lead to a gestation-dependent susceptibility to oxidative lung injury and decreased vulnerability with age consistent with the increased risk of BPD observed in preterm infants. Yet paradoxically, lethality of hyperoxia is *greater* in adults than in newborn animals of many species including rabbits, rats and mice (10, 11, 16). Supraphysiologic oxygen is lethal within days for these adult species whereas neonates survive (4, 17). The enhanced vulnerability in adult animals suggests that their cellular antioxidant capacities may be attenuated relative to the newborn lung cells.

Animal studies suggest that failed induction of antioxidant enzymes during oxygen exposure plays a greater role in adult vulnerability than baseline antioxidant activity. (18, 19) However, tolerance has not always correlated with increased antioxidant enzyme activity (20). A less favorable balance of superoxide generating capacity to antioxidant enzyme activity has also been implicated (21). To test the hypothesis that enhanced antioxidant defenses confer protection in the newborn lung while decreased defenses contribute to adult susceptibility, we used live cell imaging of a targeted redox-sensitive probe to determine subcellular oxidative responses to hyperoxia. Identifying age dependent differences in cellular responses to hyperoxia highlights variations in underlying mechanisms of adult and neonatal oxidative lung injury and the importance of developmentally designed therapeutic interventions.

MATERIALS AND METHODS

Intact Murine Lung Slices

All animal work was reviewed and approved by the Institutional Animal Care and Use Committee at Northwestern University. 200 μ m-thick lung slices were generated on a Leica Microsystems VT 1000S tissue slicer (Setzlar, Germany) from agarose inflated lung tissue of postnatal day or P5–7, P10–12 or 8–12 week old female C57/BL6 mice as previously described with slight modification (22).

Briefly, mice were anesthetized with ketamine (40 mg/kg) and xylazine (3 mg/kg), the trachea was cannulated with a 23 gauge angiocatheter and the heart and lung were removed *en bloc*. For transfection of alveolar epithelial cells (AEC), adenovirus was delivered endotracheally followed by lung inflation with 1 mL of 1.5% agarose. For pulmonary arterial smooth muscle cell (PASMC) studies, vessels were labeled for identification by injection of Cell Tracker Red (Invitrogen, Carlsbad, CA) into the right ventricle prior to agarose inflation. Lung cells were subsequently transfected via addition of adenovirus directly to the lung slice media. The intact lung slice technique and confirmatory immunostaining of labeled PASMC has been published previously by our lab (22).

For culturing, lung slices were originally generated on the vibratome in serum free M199 prior to placement into M199 complete with 10% fetal bovine serum (FBS), 40 mg/ml gentamicin, 100 units/ml penicillin, and 100 mg/ml streptomycin for 36 to 48 hours. In the slicing process, non-structural cells (i.e. intravascular and intra-alveolar cells) are released from the tissue into the slicing media before placing into culture.

Primary Alveolar Type II Cells

Alveolar epithelial type 2 (AT2) cells were isolated from male Sprague Dawley P3 and adult (200–225g) rats as previously described (23, 24). Briefly, the lungs were perfused via the pulmonary artery, lavaged and digested with elastase (30 U/ml, Worthington Biochemical, Lakewood, NJ). AT2 were purified by differential adherence to IgG pretreated dishes and viability of over 95% was confirmed by trypan blue exclusion. Cells were suspended in Dulbecco's modified Eagle's medium containing 10% FBS, 2 mM l-glutamine, 40 mg/ml gentamicin, 100 units/ml penicillin, and 100 mg/ml streptomycin and were plated on

untreated glass coverslips and cultured for 48 hours. Confirmation of cell type and purity was routinely assessed by immunofluorescence staining and confocal imaging at 48 hours. AT2 phenotype was confirmed by anti-lamellar membrane protein p180 antibody (Covance, Princeton, NJ; 1:100) and greater than 90% purity was confirmed by anti-vimentin antibody to identify fibroblasts (Covance, Princeton, NJ; 1:100).

Redox Measurement

Changes in subcellular compartmental oxidative state were measured during hyperoxia using a redox-sensitive protein sensor, RoGFP, targeted to either the mitochondrial matrix or cytosol as previously published (25–28). Briefly, this ratiometric fluorescent probe exhibits reciprocal emissions at 400 and 488nm in the oxidized and reduced state due to the reversible formation of disulfide bonds. Calibration of the probe is achieved at the end of experiments by administration of reducing (dithiothreitol (1mM)) and oxidizing agents (*tert*-butyl hydroperoxide (1mM)). Lung slices and AT2 cells were transfected with RoGFP adenovirus (3×10^7 PFU/mL, Viraquest, North Liberty, IA) at 8 μ L/lung slice, 10–15 μ L/lung endotracheally or 40 PFU/cell 36 hours prior to imaging.

Fluorescence Imaging

Live cell imaging of lung slices and AT2 cells was performed in a flow through chamber on an inverted fluorescent microscope under controlled oxygen and CO₂ conditions. Baseline oxidation was determined by superfusion with a balanced salt solution [BSS: NaCl (177 mM) KCl (4.0 mM), NaHCO₃ (18mM), MgSO₄ (0.76 mM), NaH₂PO₄ (1 mM), CaCl₂ (1.21 mM) and glucose (5.6 mM)] bubbled with 21% O₂, 5% CO₂ and 74% N₂ gas. Hyperoxic exposure was achieved by switching gas to 95% O₂, 5% CO₂. Data was collected every 60 seconds and averaged for all selected cells. Data was obtained using MetaFluor Fluorescence Ratio Imaging Software (Molecular Devices, Sunnyvale, CA).

Antioxidant Treatment

For selected studies, AT2 cells were transfected with an adenoviral construct expressing a mitochondrially targeted catalase (University of Iowa, Iowa City, IA) or control empty virus (Viraquest, North Liberty, IA) at 40 PFU/cell. In additional studies, AT2 cells or lung slices were treated with EUK134 (40 μ M), a synthetic SOD-catalase mimetic (Caymen Chemical Company, Ann Arbor, MI) 30 minutes prior to and during fluorescent imaging.

In Vivo Hyperoxia

Female C57/BL6 mice were treated with 24 hours of 75% O₂ in a Biospherix Chamber (Biospherix, Lacona NY) under standard and controlled temperature, humidity and housing conditions. Pre-weaning age mice (21 days or younger) were exposed along with littermates and the nursing dam. Whole lungs of hyperoxic treated and age matched female controls were harvested for protein and mRNA isolation. Lungs were perfused free of blood by 5–10 mL PBS flush via the right ventricle prior to harvest. For antioxidant experiments, female mice were IP injected with EUK134 (10mg/kg) or vehicle 24 and 0.5 hours prior to 24 hours of 75% O₂.

Western Blot Analysis

Lung protein was isolated from PBS perfused whole lung samples using a lysis buffer consisting of Tris HCl pH 7.4 (50mM), NaCl (150mM), Triton X-100 (1%), EDTA (2mM), β -glycerophosphate (40mM), PMSF (1mM), NaF (10mM), sodium orthovanadate (250 μ M) and a protease inhibitor cocktail (Roche, Indianapolis, IN). Protein lysates were separated on SDS-PAGE gels, transferred to nitrocellulose membranes and blotted with primary antibodies, followed by HRP conjugated secondary antibodies and ECL reagent (Amersham, Piscataway, NJ). Primary antibodies used included catalase (rabbit; Abcam, Cambridge, MA; 1:3000), GPX1 (rabbit; Cell Signaling, Danvers, MA; 1:1000), NOX1 (rabbit; Santa Cruz Biotechnology, Inc., Santa Cruz, CA; 1:500) SOD1 (rabbit; Enzo Life Sciences, Farmingdale, NY; 1:2000), SOD2 (rabbit; Enzo Life Sciences, Farmingdale, NY; 1:2000), SOD3 (rabbit; Enzo Life Sciences, Farmingdale, NY; 1:1000), β Actin HRP (rabbit; Cell Signaling, Danvers, MA; 1:2000) and GAPDH HRP (rabbit; Cell Signaling, Danvers, MA; 1:2000). Bands were analyzed using NIH Image J software. Protein expression was normalized to GAPDH or β Actin.

SOD Activity Assay

Whole lung protein was analyzed using a commercially available SOD assay kit (Stressgen, Ann Arbor, MI). Briefly, superoxide generated by xanthine oxidase converts water soluble tetrazine-1 (WST-1 or 2-(4-Iodophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium) to produce a formazan dye that absorbs light at 450 nm. SOD reduces the rate of color formation and sample activity is calculated from a standard curve using purified SOD of known activity. Cyanide inactivates SOD1 and SOD3 but has no effect on SOD2 activity. SOD2 activity can therefore be determined by incubating lysates with 2mM potassium cyanide (KCN). SOD2 and total SOD activity were normalized to protein content and expressed relative to normoxic P7 controls.

Immunohistochemistry

Lungs from P7 and 8 week old mice were inflation fixed with 4% formaldehyde under fixed pressure of 25 cm H₂O. Lungs were paraffin embedded and 5 micron thick tissue slices generated. After blocking with 5% bovine serum albumin (BSA) for an hour, samples were incubated with SOD2 and SOD3 antibodies above (see Western Blot) at 1:50 dilution overnight. Secondary labeling was performed using donkey anti rabbit IgG Alexa Fluor 488 at 1:200 for 1 hour (Life technologies, Carlsbad, CA). Imaging was performed on an epifluorescence microscope using Metamorph software (Molecular Devices, Carlsbad, CA).

Real Time PCR analysis

PBS perfused whole lung isolates were frozen and ground on liquid nitrogen. RNA was isolated using Aurum Total RNA Mini Kit (BioRad, Hercules, CA) and quantified using the Quant-it RiboGreen assay (Molecular Probes/Invitrogen, Carlsbad, CA). cDNA preparation and real-time PCR were performed using iScript cDNA Synthesis Kit, the iQ SYBR Green Supermix and iCycler iQ PCR detection system (BioRad). Primer sequences were as follows: NOX1 5'GGG CAG ATT TAC AGC ACA GCA CTT-3' and 5'TCC CAG CAT GGA GTG AAA GAG GTT-3'; GAPDH 5'-TTC ACC ACC ATG GAG AAG GC-3' and

5'-GGC ATG GAC TGT GGT CAT GA-3'. A single product was confirmed on melt-curve analysis with good correlation for efficiency and standard curves ($r^2 = 0.98$). Relative quantity of NOX1 mRNA was normalized to GAPDH expression using the cycle threshold ($-CT$) method (29). For each reaction, negative controls containing reaction mix and primers without cDNA were performed to verify that primers and reaction mixtures were free of template contamination.

Cell Death Assays

Intact murine lungs slices were generated from 7 day old and 8–12 week old female mice as described above. Lung slices were subsequently exposed to 95% O₂/5% CO₂ or 21 % O₂/5% CO₂ for 48 hours. The culture media was treated with placebo or EUK134 (40 μ M) at 0 and 24 hours of exposure without changing of media. Cell death was determined using a lactate dehydrogenase (LDH) assay (Biovision, San Francisco, CA). Using an enzymatic coupling reaction, LDH is quantified in the supernatant and the tritonized lung slice lysate. LDH activity is determined by spectrophotometric reading at OD₄₉₀. Percent cell death is calculated as $\text{supernatant}/(\text{supernatant} + \text{lysate}) \times 100$.

Statistics

All data are expressed as mean $\pm$ standard error of the mean. For RoGFP oxidation experiments, n represents the average of all cell responses on a lung slice or coverslip. When comparing two conditions, experiments were carried out on the same day to control for differences in hyperoxic response of cultured AT2 cells and lung slices. Results were analyzed using a 2-way ANOVA with Bonferonni *post hoc* analysis. For protein, mRNA and enzyme activity studies, a 1-way ANOVA with *post hoc* comparison by Neuman-Keuls were used. All data was analyzed using Prism software (Graph-Pad Software Inc., San Diego, CA) with statistical significance defined as $p < 0.05$.

RESULTS

Studies of lung homogenates, cultured cells and isolated organelles suggest that hyperoxia amplifies ROS generation within the mitochondria (30–35). However, subcellular oxidant stress at different developmental stages in specific lung cells has not been directly assessed. We therefore used a targeted ratiometric redox-sensitive probe, RoGFP, to assess subcellular responses to acute hyperoxia in cultured lung epithelial cells and in cells within intact lung slices.

Alveolar epithelial cells of intact lung slice

We began by assessing oxidant stress in the mitochondrial matrix. Intact lung slices of mice at P5–7, P10–12, P21 and 8–12 week old adults were compared during acute exposure to hyperoxia. Mitochondrial oxidative stress responses to acute hyperoxia within AEC of lung slices from P5–7 and P10–12 mice were exaggerated as compared with adult lung cells (Fig. 1A). The increase in matrix oxidation in P5–7 and P10–12 cells was significantly greater than in adult cells as assessed by repeated measures ANOVA. Moreover, statistically significant increases from baseline were detected earlier for P5–7 and P10–12 cells than for adult AEC. When P21 AEC were compared with adult cells, no statistical differences were

detected (data not shown). Figure 1B demonstrates a representative fluorescence image of RoGFP expression within epithelial cells of a lung slice.

We then assessed the oxidative stress response to acute hyperoxia using RoGFP expressed in the cytosol. Alveolar epithelial cells of P7 and adult lungs were compared (Fig. 2A). Both P5–7 and 8w AEC failed to demonstrate a detectable increase in cytosolic RoGFP oxidation during 30 minutes of hyperoxia. A progressive decrease in oxidation was observed in both groups over time, which could suggest deterioration of the sensor in hyperoxia. We therefore assessed the RoGFP response during normoxic exposure. As shown in Fig. 2B, a similar tendency for the probe to reduce was observed, suggesting that this was not the case. Conceivably this response could represent an adaptation of the cells to serum-free conditions or other changes inherent to the experimental environment.

Isolated type II epithelial cells

The alveolar epithelial cells studied in the lung slice experiments would include both Type I and Type II cells. Compared with Type I, Type II cells exhibit fewer ultrastructural abnormalities after hyperoxic exposure *in vivo* (14, 36, 37), suggesting that they may be less vulnerable to hyperoxia-induced oxidative stress. We therefore compared oxidative stress responses to hyperoxia in primary alveolar Type II cells from P3 and adult rats. In Type II cells from P3 rats, the increase in oxidation during hyperoxia was significantly greater compared with adult cells, as assessed by repeated measures ANOVA (Fig. 3A). Under baseline normoxic conditions, the adult cells tended to show a greater degree of mitochondrial matrix oxidation ($p > 0.05$). To assess the purity and phenotypic identity of the P3 Type II cells, immunofluorescence studies using antibodies to p180 (specific for Type II) and vimentin (specific for fibroblasts) were performed (Fig. 3B). Analysis revealed that >90% of the cells exhibited a Type II phenotype 48 hr after isolation, the time at which the RoGFP studies were carried out. Similar retention of the Type II phenotype at 48 hr was observed in adult cells (not shown).

PASMC of intact lung slice

To assess the cell-type specificity of oxidant stress responses, hyperoxia experiments were repeated in pulmonary artery smooth muscle cells (PASMC) within lung slices from newborn and adult mice. In contrast to alveolar epithelial cells, the mitochondrial RoGFP responses to hyperoxia did not differ between the P7 and adult PASMC (Fig. 4A). A representative fluorescence image of a vessel identified as a pulmonary artery in a lung slice is also shown (Fig. 4B)

Isolated type II cell plus mitochondrially targeted antioxidant

Hyperoxia enhances superoxide generation from the electron transport chain. Superoxide dismutase (SOD2) degrades this radical to H_2O_2 , which is then detected by the RoGFP sensor. To confirm the involvement of H_2O_2 , we over-expressed catalase within the mitochondria of adult rat Type II cells to diminish its contribution. Expression of mitochondrially targeted catalase (mitoCAT) attenuated hyperoxia-induced mitochondrial oxidation compared with empty vector controls (Fig. 5A), as determined by repeated measures ANOVA. Notably, the baseline mitochondrial RoGFP oxidation was greater in

these cultured cells than in epithelial cells studied in lung slices (Fig. 1A). One concern was that the Type II cells were treated with two adenoviruses (RoGFP plus mitoCAT or empty virus) whereas the slices were only treated with one. We therefore assessed the response in adult Type II cells treated with a chemical antioxidant, EUK 134, and a single adenovirus encoding mito-RoGFP. Baseline oxidation in those cells was still greater than in the lung slices, but tended to be less than in the mitoCAT experiments. Nevertheless, EUK 134 attenuated the mitochondrial stress response to hyperoxia to a similar extent (Fig. 5B), underscoring the importance of matrix H₂O₂ involvement.

In vivo: whole lung antioxidant expression

While the embryonic developmental pulmonary antioxidant enzyme expression has been characterized in a range of species (10), less data exists regarding postnatal maturation. To determine whether altered antioxidant expression contributes to the developmental differences we observed in response to hyperoxia, whole lung protein lysates from P7, P21 and 8 week old mice were compared via Western blot analysis. Compared with P7 lungs, protein expression of glutathione peroxidase 1 (GPX1) and SOD2 and SOD3 increased in p21 and 8 wk lungs. SOD1 also increased at 8 wk, but no increase was detected in P21 lungs (Fig. 6A). Representative immunohistochemistry results are also shown (Fig. 6B). These results suggest that newborn lungs express lower levels of antioxidant enzymes compared with older animals, which may explain the enhanced oxidant stress we observed in response to hyperoxia.

The enhanced mitochondrial oxidant stress, combined with the relatively lower antioxidant expression in the P7 lungs suggested that the lungs of younger animals should be more vulnerable to hyperoxia-induced oxidant stress. Yet mortality in response to hyperoxia is lower in newborn mice compared with adults. We therefore compared the *in vivo* response to hyperoxia in P7 and 8 wk old mice. Whole lung protein lysates of P7 and 8 wk mice exposed to 75% oxygen were analyzed. No significant increase in catalase or SOD1–3 expression was detected in either the newborn or adult lung after 24 hours of hyperoxia (data not shown). Total SOD activity was greater in the adult lungs compared with P7 mice (Fig. 7A), consistent with the higher SOD protein expression levels (Fig. 6A). Adult mice demonstrated a small but significant increase in total SOD activity during hyperoxia that was not observed in P7 mice (Fig. 7A). This was not accompanied by any change in mitochondrial SOD2 activity (Fig. 7B).

In vivo: Whole lung NADPH oxidase 1 expression

While mitochondria represent an important source of ROS generation during hyperoxia, other oxidases can potentially contribute. We therefore examined the expression of the superoxide generating enzyme, NADPH oxidase 1 (NOX1) during baseline and after 24 hr of hyperoxia in P7 and adult mice. Compared with P7 lungs, adult mice exhibited increased NOX1 mRNA and protein expression under normoxic baseline conditions (Fig. 8). After 24 hours of hyperoxia, significant increases were noted in the P7 animals but not in the adults. To determine if this response is regulated by ROS production, P7 mice were treated with EUK134 or vehicle prior to hyperoxic exposure. The increase in NOX1 expression during 24 hr of hyperoxia was attenuated by treatment with EUK134, indicating that ROS signals

are involved in the upregulation of NOX1 in the P7 animals (Fig. 9). P7 lung slices treated with EUK134 demonstrated attenuated increases in RoGFP oxidation during acute hyperoxia, confirming that EUK 134 treatment is effective in limiting hyperoxia-induced mitochondrial oxidant stress (Fig. 9C).

Intact lung slice and cell death

To determine if the increased ROS response to hyperoxia in P7 lungs corresponded with an increased susceptibility cell death, lung slices from P7 and adult mice were treated with EUK134 or vehicle and exposed to 48 hr of hyperoxia (95% O₂ and 5% CO₂). LDH assays demonstrated a significant increase in cell death during hyperoxia in the P7 but not in adult lungs (Fig. 10). While EUK134 attenuated cell death in control lung slices, treatment did not protect against hyperoxia-mediated cell death as loosely assayed by LDH. These results suggest that hyperoxia augments cell death to a greater extent in the P7 than the adult lung slices.

DISCUSSION

We employed novel techniques including subcellular targeted redox sensors, live cell imaging, and intact lung slices to investigate developmental differences in cellular responses and susceptibility to supraphysiologic oxygen. Newborn mouse models are well suited for this work as murine alveolar development begins on postnatal day 3 with completion of sacculation by P14 (38). As a result, studies in P5–7 mice parallel the structural development of an early term infant (36 weeks gestation) and P10–12 that of a full term infant lung. Our data using compartmentalized redox sensors in alveolar epithelial cells reveal that the mitochondrial oxidative responses to acute hyperoxia were greater in young animals compared with adults.

This difference could conceivably have been caused by a lower expression of antioxidant enzymes in the younger animals compared with adults. Indeed, we observed that protein expression levels for several antioxidant enzymes including mitochondrial SOD2 were lower in the P7 lungs, and increased with further development. Moreover, we found that cell death during 48 hours of hyperoxia was greater in lung slices from P7 animals as compared with adults. While our data are internally consistent, these findings were surprising given that hyperoxia-induced lung injury and lethality is greater in adults across multiple species compared to newborns (10). This age dependent difference in the oxidative stress response was even present in the more resistant alveolar type II cells.

One mechanism to explain why our cellular responses contrast with the enhanced newborn tolerance to hyperoxia observed *in vivo* may relate to the newborn's ability to augment antioxidant expression in response to oxidant stress as suggested in other models (17, 18, 21, 39, 40). To test this question we measured the induction of antioxidant enzymes during 24 hr hyperoxia. However, these studies indicated that P7 lungs failed to induce SOD activity or antioxidant expression during acute hyperoxia, indicating that this mechanism may not explain differences. Studies which have attributed neonatal tolerance to AOE expression differed from our work in the concentration of oxygen employed, age at exposure and

species studied (11, 16–19). While our studies used 95% oxygen for *in vitro* experiments, *in vivo* exposures were limited to 75% oxygen to avoid maternal morbidity.

Another possible explanation for the disparity between our data and previous work relates to the duration and degree of hyperoxic exposure. Hyperoxic lung injury in the adult typically develops over several days, and the present study only evaluated responses to shorter exposures. Although hyperoxic lung injury has been linked to oxidative stress (41), the magnitude of that stress and the subcellular compartments affected by prolonged hyperoxia may differ between the newborn and the adult. In that regard, prolonged hyperoxia might permit leakage of oxidants from the mitochondria to the cytosol to a different extent in newborns and adults. A better ability to contain oxidants in the matrix of the newborn could then protect other compartments from stress despite the greater level of matrix oxidation. Acute oxidative stress responses in newborn animals might also trigger protective mechanisms such as the activation or recruitment of lung progenitor cells that could attenuate lung injury or enhance lung repair. Further studies examining responses to prolonged hyperoxia may shed light on these mechanisms.

While the acute exposure was insufficient to observe antioxidant induction, we detected augmented NOX1 expression with 24 hr hyperoxia in the P7 but not adult lung. NOX1 has been identified as an important mediator of hyperoxic lung injury (42, 43). Diphenyleneiodonium (DPI), an inhibitor of virtually all oxidoreductases in the cell, has been reported to decrease ROS generation and cell death in pulmonary epithelial and endothelial cells challenged with hyperoxia (15, 44, 45). *In vivo* studies using NOX1 knockout mice demonstrated protection against hyperoxic lung injury and ROS generation, whereas knockout of NOX2 failed to confer protection (42). Although lungs from adult mice showed higher levels of NOX1 mRNA and protein expression under normoxic conditions compared with P7 animals, no difference in cytosolic oxidation were detected (Fig. 2). After 24 hr exposure to hyperoxia, the P7 lung demonstrated induction of NOX1 message and protein, but this induction was absent in adults. As NOX1 contributes to ROS generation in the cytosol, its activity could contribute to the amplification of oxidant stress beyond the mitochondria. Indeed, the antioxidant EUK134 attenuated hyperoxia-induced increases in NOX1 expression, indicating that its induction is mediated by oxidant stress signals. NOX1 transcriptional activation is regulated by Activating Transcription Factor-1 (ATF-1), which is triggered by Platelet-Derived Growth Factor (PDGF) and Prostaglandin F_{2α} in a mitochondria-dependent manner (43). These data suggest that the oxidative stress in the newborn lung involves two components – an exaggerated mitochondrial oxidation in response to acute hyperoxia, and a ROS-mediated induction of NOX1 expression that may contribute to the later amplification of ROS in compartments other than the mitochondria.

The disparity between an increased oxidative stress response, NOX1 induction and greater cell death observed in the P7 lung and neonatal tolerance of hyperoxia could reflect the contributions of inflammation *in vivo* that do not occur *in vitro*. Hyperoxic lung injury leads to recruitment of inflammatory cells, with subsequent release of inflammatory mediators resulting in local tissue damage (1–3). As recruitment of inflammatory cells does not occur in the lung slice model, the contributions of that response are underestimated. Developmental differences in hyperoxia-induced lung inflammation could explain neonatal

tolerance as inflammatory responses in the neonatal lung are delayed (46–49). In addition, neonatal inflammatory cells recruited in hyperoxia release less enzymatic factors resulting in reduced tissue injury (50). Differences in type and temporal sequence of cytokine release and transcription factor activation has been reported, with early IL-1 and IL-6 release in the adult lung contributing to inflammation-mediated injury (51). Maturation differences in activation of NF κ B and AP1 may also influence inflammation and cellular apoptosis in hyperoxia (52, 53). Hence, the greater adult susceptibility observed *in vivo* but not *in vitro* may be attributed to a more robust inflammatory response. Similarly, contributions made by bone marrow-derived progenitor cells, hormonal and humoral factors are absent in the lung slice model and are known to modulate hyperoxic lung injury (5, 54, 55).

Alveolar epithelial cells experience higher levels of oxygen than other lung cells, and their response to hyperoxia may influence the degree of lung injury. Some controversy exists regarding the ability of SOD2 to mitigate lung injury in response to hyperoxia. Previous studies of mice carrying a transgene to express SOD2 under the control of the β -actin promoter failed to prolong survival of mice under 99% O₂. (56) However, we previously demonstrated that amplified expression of SOD2 in alveolar epithelial cells confers protection against hyperoxia in adult mice. (13) In addition, studies in transgenic mice carrying a human SOD2 driven by a surfactant protein C (SP-C) promoter also demonstrated protection (57). These findings suggest that mitochondrial superoxide scavenging in the epithelium plays an important role in modulating hyperoxic lung injury. The total SOD activity in P7 lungs was less than in adults, although SOD2 activity was not different. Immunofluorescence staining of SOD2 and the extracellular SOD3 suggest that postnatal maturation of these antioxidants occurs across many lung cell types, including airway, alveolar and vascular cells. However, it was not possible to quantify the relative cell type-specific increases from these images. Whole lung responses reflect contributions from many different cell types, each of which may respond differently to hyperoxia. For example, no developmental differences in the mitochondrial oxidant response to hyperoxia were detected in smooth muscle cells in the pulmonary artery wall (Fig. 4A). It seems likely that the survival of animals in hyperoxia depends on the effective scavenging of ROS in multiple cell types, so the developmental differences in the oxidative stress response we observed during short-term exposure in epithelial cells may not correlate with overall lethality during more prolonged exposure.

In conclusion, this study defines subcellular compartmental responses to hyperoxia during postnatal lung development. Using a lung slice model, the data implicate enhanced mitochondrial matrix oxidant stress in the P7 lung compared with adults during acute hyperoxia. In addition, we find developmental differences in the lung cellular response to hyperoxia with respect to antioxidant enzyme expression, NOX1 induction and cell death. These differences may contribute to the distinct developmentally influenced phenotypes observed in hyperoxia-induced lung injury, with the immature lung demonstrating compromised alveolar and vascular development. Therapies for neonatal and adult oxidative lung injury should therefore consider and address developmental differences in oxidative stress responses.

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ABBREVIATIONS USED

AEC	alveolar epithelial cells
ANOVA	analysis of variance
AOE	antioxidant enzymes
AT2	alveolar type 2
BPD	bronchopulmonary dysplasia
NOX1	NADPH oxidase 1
LDH	lactate dehydrogenase
P#	postnatal day
PASMC	pulmonary artery smooth muscle cells
PDGF	platelet derived growth factor
PGF_{2α}	prostaglandin F _{2α}
ROS	reactive oxygen species

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HIGHLIGHTS

- Acute hyperoxia increases oxidative stress within the mitochondrial matrix but not the cytosol
- Neonatal alveolar cells exhibit exaggerated hyperoxia-induced mitochondrial oxidant stress
- Lung slices from newborn mice demonstrate increased hyperoxia-induced cell death
- *In vivo* hyperoxia induces NOX1 mRNA and protein expression in newborn but not adult murine lung
- NOX1 induction is ROS-dependent and attenuated by treatment with an antioxidant
- Alveolar epithelial cells of newborn mice demonstrate exaggerated hyperoxia-induced mitochondrial matrix oxidant stress compared to adults
- 24 hours of hyperoxia *in vivo* induces NOX1 mRNA and protein expression in newborn but not adult murine lung

FIGURE 1.

FIGURE 1A,B: 30 Minutes of Hyperoxia Induces Exaggerated Oxidative Stress Response in AECs of Immature Mouse Lung Slices when Compared to Adult

A. The redox-sensitive protein sensor, RoGFP, was expressed in the mitochondrial matrix of AEC of lung slices generated from P5–7, P10–12 and 8 week old adult mice. Superfusion with hyperoxic media resulted in increased oxidation in the mitochondrial matrix within minutes of exposure. Younger AECs demonstrated exaggerated mitochondrial oxidation as compared to adult. Significant increase above baseline was reached at 11, 17 and 25 minutes for P5–7, P10–12 and 8 week old AECs with $p < 0.05$ represented as *, ** and † respectively. Data are presented as mean $\pm$ SEM. $N = 6$ or greater. B. Representative fluorescence image of RoGFP expression in AEC of lung slice.

FIGURE 2.

FIGURE 2A,B: 30 Minutes of Hyperoxia Fails to Induce Cytosolic Oxidative Stress Response in AEC of P5-7 and Adult Mouse Lung Slices

A. AEC of P5-7 and adult lung slices expressing cytosolic RoGFP were superfused with hyperoxic media. Neither P5-7 nor mature AEC demonstrated increased cytosolic oxidative stress with 30 minutes of hyperoxia. N = 5. B. AEC expressing cytosolic RoGFP exposed to 30 minutes of normoxia, N=3.

FIGURE 3.

FIGURE 3A,B: 30 Minutes of Hyperoxia Induces Exaggerated Oxidative Stress Response in P3 Rat Alveolar Type II Cells

A. Type II cells isolated from P3 rats demonstrated significantly greater increase in hyperoxia-induced mitochondrial oxidation when compared to adult type II cells. Significant increase above baseline oxidation was reached at 7 and 24 minutes for P3 and adult cells respectively, with $p < 0.05$ represented as * and †. Data are presented as mean $\pm$ SEM. N = 8 or greater. B. Representative immunofluorescent images of alveolar cells isolated from P3 rats. Type II phenotype is confirmed by P180 staining and purity assessed by staining for vimentin.

FIGURE 4.

FIGURE 4A,B: 30 Minutes of Hyperoxia Induces Comparable Oxidative Stress Response in P7 and Adult PASM of Mouse Lung Slices

PASM of P7 and adult lungs slices demonstrate comparable mitochondrial oxidative stress response with exposure to hyperoxia. Data are presented as mean $\pm$ SEM. N = 5 or greater.

B. Representative fluorescence image of RoGFP expression in PASM of lung slice.

FIGURE 5.

FIGURE 5A,B: Mitochondrial Oxidation is Attenuated by Mitochondrial Catalase and EUK134 in Rat Alveolar Type II Cells

A. Over expression of mitochondrial catalase (mitoCAT) attenuated hyperoxia-induced mitochondrial oxidation in adult rat type II cells. Significant increase above baseline was achieved at 14 and 18 minutes for empty virus (control) and mitoCAT expressing cells respectively, with $p < 0.05$ represented as * and †. Data are expressed as mean $\pm$ SEM. $N = 8$ or greater. B. The chemical antioxidant EUK134 attenuates hyperoxia-induced mitochondrial oxidation in adult type II cells. $p < 0.05$ is represented as * and † respectively. Data are expressed as mean $\pm$ SEM. $N = 4$.

FIGURE 6.

FIGURE 6A,B: Postnatal Maturation of Antioxidant Expression in the Murine Lung
 (A) Whole lung protein isolated from P7, P21 and 8w mice demonstrate postnatal maturation of pulmonary antioxidant expression with significant increase in GPX1, SOD1,2 and 3 in adult lung and GPX1, SOD2 and 3 in P21 compared to P7 as indicated by * for $p < 0.05$. Only SOD1 and SOD2 were significantly increased in 8w compared to P21 as indicated by † for $p < 0.05$. Data are presented as mean + SEM. $n = 6$ for each group. (B) Representative SOD2 and SOD3 immunohistochemistry images of P7 and adult lungs.

FIGURE 7.

FIGURE 7A,B: 24 Hours of Hyperoxia Induces Increased SOD Activity in Adult but Not P7 Mouse Lung

Whole lung protein isolated from P7 and 8w mice exposed to 24 hours of 75% O₂ was compared to controls. A. Hyperoxia induced increased SOD activity in adults without change in the P7 lung. * $p < 0.05$ as compared to normoxic control. B. Hyperoxia failed to induce an increase in SOD2 activity in adult or P7 lung. Data is presented as mean + SEM. $n = 6$.

FIGURE 8.

FIGURE 8A, B, C: 24 Hours of Hyperoxia Induces Increased NOX1 mRNA and Protein Expression in P7 but Not Adult Mouse Lung

Whole lung mRNA and protein isolated from P7 and 8w mice exposed to 24 hours of 75% O₂ was compared to normoxic controls. NOX1 mRNA (A) and protein expression (B) increased significantly in hyperoxic-exposed P7 without change in the adult lung. * $p < 0.05$ as compared to normoxic control. Data are presented as mean + SEM. $n = 7$ or greater for each group. (C) A representative gel.

FIGURE 9.

FIGURE 9A, B, C: EUK134 Attenuates Hyperoxia-Induced Increase in NOX1 Expression in P7 Mouse Lung

(A) P7 mice were injected with placebo or EUK134 prior to 24 hours of 21% or 75% O₂. Analysis of whole lung protein isolates demonstrates attenuated hyperoxia-induced increase in NOX1 expression by EUK134. * $p < 0.05$ as compared to normoxic vehicle control. † $p < 0.05$ as compared to hyperoxic vehicle control. $n = 6$ or greater. Data are presented as mean $\pm$ SEM. (B) A representative gel. (C) In P7 lung slices, 30 minutes of hyperoxia induces increased mitochondrial oxidation which is attenuated by treatment with EUK134. $p < 0.05$ is represented as * and † as compared to normoxic vehicle and hyperoxic vehicle respectively. Data are expressed as mean $\pm$ SEM. $N = 5$.

FIGURE 10.

FIGURE 10A: 48 Hours of Hyperoxia Induces Increased Cell Death in P7 but not Adult Murine Lung Slices

A. Lungs slices generated from P7 and adult mice were treated with EUK134 or vehicle and exposed to 48 hours of 95% O₂/5% CO₂ (hyperoxia). P7 but not adult lung slices demonstrated increased hyperoxia-induced cell death by LDH assay as compared to 21% O₂/5% CO₂ (normoxia). EUK134 attenuated baseline and hyperoxia-induced cell death as compared to placebo treated. $p < 0.05$ is represented as *. Data are presented as mean + SEM. N = 5 with each n representing the average of three slices generated from one mouse.