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Original Contribution

Developmental differences in hyperoxia-induced oxidative stress and cellular responses in the murine lung

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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 h 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 h of *in vivo* exposure was absent. However, expression of the reactive oxygen species-generating enzyme NADPH oxidase 1 was increased with hyperoxic exposure in the young but not the 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 in antioxidant defenses. Therapies for neonatal and adult oxidative lung injury should therefore consider and address developmental differences in oxidative stress responses.

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. Although 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 (AOEs) 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

Abbreviations: AEC, alveolar epithelial cell; AOE, antioxidant enzyme; AT2, alveolar type 2; BPD, bronchopulmonary dysplasia; NOX1, NADPH oxidase 1; LDH, lactate dehydrogenase; P, postnatal day; PASMC, pulmonary artery smooth muscle cell; ROS, reactive oxygen species.

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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 and 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. Lung slices 200 μm thick were generated on a Leica Microsystems VT 1000S tissue slicer (Setzlar, Germany) from agarose-inflated lung tissue of postnatal day (P) 5–7, P10–12, or 8- to 12-week-old female C57BL/6 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 (AECs), 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 CellTracker red (Invitrogen, Carlsbad, CA, USA) into the right ventricle before agarose inflation. Lung cells were subsequently transfected via addition of adenovirus directly to the lung slice medium. The intact lung slice technique and confirmatory immunostaining of labeled PASMCs have been published previously by our lab [22].

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

Primary alveolar type II cells

Alveolar epithelial type 2 (AT2) cells were isolated from male Sprague–Dawley P3 and adult (200–225 g) 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, USA). AT2 cells 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 U/ml penicillin, and 100 mg/ml streptomycin and were plated on untreated glass coverslips and cultured for 48 h. Confirmation of cell type and purity was routinely assessed by immunofluorescence staining and confocal imaging at 48 h. AT2 phenotype was confirmed by anti-lamellar membrane protein p180 antibody (Covance, Princeton, NJ, USA; 1:100) and greater than 90% purity was confirmed by anti-vimentin antibody to identify fibroblasts (Covance; 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 the cytosol as previously published [25–28]. Briefly, this ratiometric fluorescent probe exhibits reciprocal emissions at 400 and 488 nm in the oxidized and reduced state because of the reversible formation of disulfide bonds. Calibration of the probe is achieved at the end of experiments by administration of reducing (dithiothreitol, 1 mM) and oxidizing agents (*tert*-butylhydroperoxide, 1 mM). Lung slices and AT2 cells were transfected with RoGFP adenovirus (3×10^7 pfu/ml; Viraquest, North Liberty, IA, USA) at 8 μl/lung slice, 10–15 μl/lung endotracheally, or 40 pfu/cell 36 h before imaging.

Fluorescence imaging

Live-cell imaging of lung slices and AT2 cells was performed in a flow-through chamber on an inverted fluorescence microscope under controlled oxygen and CO_2 conditions. Baseline oxidation was determined by superfusion with a balanced salt solution (NaCl (177 mM), KCl (4.0 mM), $NaHCO_3$ (18 mM), $MgSO_4$ (0.76 mM), NaH_2PO_4 (1 mM), $CaCl_2$ (1.21 mM), and glucose (5.6 mM)) bubbled with 21% O_2, 5% CO_2, and 74% N_2 gas. Hyperoxic exposure was achieved by switching the gas to 95% O_2, 5% CO_2. Data were collected every 60 s and averaged for all selected cells. Data were obtained using MetaFluor Fluorescence Ratio imaging software (Molecular Devices, Sunnyvale, CA, USA).

Antioxidant treatment

For selected studies, AT2 cells were transfected with an adenoviral construct expressing a mitochondrially targeted catalase (University of Iowa, Iowa City, IA, USA) or control empty virus (Viraquest) at 40 pfu/cell. In additional studies, AT2 cells or lung slices were treated with EUK134 (40 μM), a synthetic superoxide dismutase (SOD)–catalase mimetic (Cayman Chemical Co., Ann Arbor, MI, USA), 30 min before and during fluorescence imaging.

In vivo hyperoxia

Female C57/BL6 mice were treated with 24 h of 75% O_2 in a Biospherix chamber (Biospherix, Lacona, NY, USA) 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 phosphate-buffered saline (PBS) flush via the right ventricle before harvest. For antioxidant experiments, female mice were injected intraperitoneally with EUK134 (10 mg/kg) or vehicle 24 and 0.5 h before 24 h of 75% O_2.

Western blot analysis

Lung protein was isolated from PBS-perfused whole lung samples using a lysis buffer consisting of Tris–HCl, pH 7.4 (50 mM), NaCl