Toxicology and Applied Pharmacology 272 (2013) 281–290

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

# Sex-specific differences in hyperoxic lung injury in mice: Implications for acute and chronic lung disease in humans

Krithika Lingappan [a,*], Weiwu Jiang [a], Lihua Wang [a], Xanthi I. Couroucli [a], Roberto Barrios [b], Bhagavatula Moorthy [a]

[a] Department of Pediatrics, Section of Neonatology, Texas Children's Hospital, Baylor College of Medicine, 1102 Bates Avenue, MC: FC530.01, Houston, TX 77030, USA
[b] Department of Pathology and Genomic Medicine, The Methodist Hospital Physician Organization, 6565 Fannin Street, Suite M227, Houston, TX 77030, USA

ARTICLE INFO

*Article history:*
Received 23 March 2013
Revised 7 June 2013
Accepted 10 June 2013
Available online 21 June 2013

*Keywords:*
Hyperoxia
Sex-differences
Lung injury
Oxidative stress
Inflammation
Cytochrome P4501A1

ABSTRACT

Sex-specific differences in pulmonary morbidity in humans are well documented. Hyperoxia contributes to lung injury in experimental animals and humans. The mechanisms responsible for sex differences in the susceptibility towards hyperoxic lung injury remain largely unknown. In this investigation, we tested the hypothesis that mice will display sex-specific differences in hyperoxic lung injury. Eight week-old male and female mice (C57BL/6J) were exposed to 72 h of hyperoxia ($FiO_2 > 0.95$). After exposure to hyperoxia, lung injury, levels of 8-iso-prostaglandin $F_2$ alpha (8-iso-PGF 2α) (LC–MS/MS), apoptosis (TUNEL) and inflammatory markers (suspension bead array) were determined. Cytochrome P450 (CYP)1A expression in the lung was assessed using immunohistochemistry and western blotting. After exposure to hyperoxia, males showed greater lung injury, neutrophil infiltration and apoptosis, compared to air-breathing controls than females. Pulmonary 8-iso-PGF 2α levels were higher in males than females after hyperoxia exposure. Sexually dimorphic increases in levels of IL-6 (F > M) and VEGF (M > F) in the lungs were also observed. CYP1A1 expression in the lung was higher in female mice compared to males under hyperoxic conditions. Overall, our results support the hypothesis that male mice are more susceptible than females to hyperoxic lung injury and that differences in inflammatory and oxidative stress markers contribute to these sex-specific dimorphic effects. In conclusion, this paper describes the establishment of an animal model that shows sex differences in hyperoxic lung injury in a temporal manner and thus has important implications for lung diseases mediated by hyperoxia in humans.



## Introduction

Sex-specific differences exist in various forms of organ injury in adults and children. Acute respiratory distress syndrome (ARDS) is a devastating clinical disorder in critically ill patients with a high mortality. Mortality in ARDS was higher in males compared to females (Agarwal et al., 2006; Moss and Mannino, 2002). Neonatal outcomes for males are worse than females for many diseases, including bronchopulmonary dysplasia (BPD). The incidence of BPD is lower among preterm girls after adjusting for other confounders (Stevenson et al., 2000). Male sex is considered an independent predictor for the development of BPD (Kraybill et al., 1989). The lung function in boys both in the neonatal period and at 1 year of age was noted to be worse when compared to girls (Stocks et al., 1997; Thomas et al., 2006). The reasons behind this are not known though better antioxidant defense mechanism in girls is thought to contribute to this advantage (Hamon et al., 2011; Vento et al., 2009a).

Oxygen toxicity is thought to play a role in both acute lung injury and BPD. Exposure to high concentrations of oxygen (hyperoxia) leads to pathological changes similar to ARDS in mammalian species (Bryan and Jenkinson, 1988; Budinger et al., 2011; Clark and Lambertsen, 1971; Freeman and Crapo, 1981; Matute-Bello et al., 2008) and prolonged exposure of newborn mice to hyperoxia leads to lung pathology similar to human BPD (Warner et al., 1998). In critically ill patients, hyperoxia may exacerbate or even cause acute lung injury (ALI). In the acute phase, after exposure to hyperoxia, lung epithelial injury and neutrophilic infiltration are observed. Exposure to hyperoxia postnatally is thought to contribute to the development of BPD in neonates (Vento et al., 2009b). 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 (Freeman and Crapo, 1981).

The cytochrome P450 (CYP) enzymes belong to a super family of hemeproteins, involved in the metabolism of exogenous and endogenous

*Abbreviations:* BPD, bronchopulmonary dysplasia; ALI, acute lung injury; ROS, reactive oxygen species; ARDS, acute respiratory distress syndrome; 8-iso-PGF2α, 8-iso-prostaglandin $F_2$alpha; CYP, Cytochrome P450.

* Corresponding author. Fax: +1 832 825 3204.
*E-mail address:* lingappa@bcm.edu (K. Lingappan).

0041-008X/$ – see front matter © 2013 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.taap.2013.06.007

chemicals (Guengerich, 1990). Induction of the CYP family of proteins has been implicated in the potentiation of hyperoxic lung injury (Hazinski et al., 1995), but on the other hand, we have demonstrated the protective effect of CYP1A enzymes (Couroucli et al., 2011; Jiang et al., 2004; Sinha et al., 2005).

The impact of sex and sex hormones on lung physiology and disease has been extensively studied in animal models. Gender also contributes to differential lung development, and has a major role in the causation of disease conditions from the neonatal (respiratory distress syndrome, BPD) to the adult period (asthma, lung cancer, interstitial lung disease) (Carey et al., 2007). This is probably due to modulation by sex hormones that may contribute to the disease pathogenesis or serve as protective factors, depending on the disease involved. With respect to acute lung injury due to hyperoxia it has been showed that castration prolonged tolerance of young male rats to pulmonary oxygen toxicity (Neriishi and Frank, 1984). In other acute lung injury models, testosterone was found to increase (Card et al., 2006) and estrogen to ameliorate inflammation and injury (Speyer et al., 2005). However, there are no studies on the temporal effects of exposure to hyperoxia on lung injury in a sex-specific model in adult mice. The sex based dimorphic response in lung injury due to hyperoxia and the differences in inflammatory and oxidative stress markers have also not been studied. In this investigation, we tested the hypothesis that males are more susceptible to hyperoxic lung injury than females and that sex-specific differences in inflammatory and oxidative stress markers contribute to the gender differences under hyperoxic conditions.

## Material and methods

*Animals.* This study was conducted in accordance with the federal guidelines for the human care and use of laboratory animals, and was approved by the Institutional Animal Care and Use Committee (IACUC) of Baylor College of Medicine. Breeding pairs of mice were obtained from the Jackson Laboratory (Bar Harbor, ME). Eight-week old male (C57BL/6J) mice were maintained at Texas Children's Hospital animal facility and used for the study. They were fed standard mice food and water ad libitum. Animals were maintained in a12-h day/night cycle. Briefly, we used a total of 20 animals per sex in the study. The mice were maintained in either room air (21% oxygen) or exposed to hyperoxia (95–100% oxygen) environment using pure $O_2$ at 5 l/min for 72 h in a sealed Plexiglas chamber, as reported previously (Gonder et al., 1985). After sealing the chamber, the oxygen concentration in the Plexiglas chamber was measured frequently by an analyzer (Getronics, Kenilworth, New Jersey). Purified tap water and food (Purina Rodent Lab Chow 5001 from Purina Mills, Inc., Richmond, IN) were available ad libitum. After 72 h of hyperoxia exposure, the animals were anesthetized with 200 mg/kg of sodium pentobarbital (i.p.) and euthanized by exsanguination while under deep pentobarbital anesthesia. The lung tissues were harvested for further analysis.

*Chemicals.* Tris, sucrose, bovine serum albumin were purchased from Sigma Chemical Co. (St. Louis, MO). All RT-PCR reagents were from Applied Biosystems (Foster City, CA). 8-Iso-PGF2α and 8-iso-PGF2α-d4 were purchased from Cayman Chemical Co. (Ann Arbor, MI).

*Lung wet weight and body weight.* The mice were weighed immediately after being anesthetized, and the lungs were weighed after the sacrifice and harvesting.

*Preparation of tissues for histology, histopathology and immunohistochemistry.* Tracheotomy was performed on the anesthetized mice and the lung tissue was fixed by intratracheal instillation of 10% zinc formalin at constant pressure of 25 cm of $H_2O$ (Couroucli et al., 2002). Samples were left in solution for 24 h in formaldehyde, and then transferred to 70% ethanol for long-term storage. Routine histology was performed on lung tissues from individual animals following staining of the paraffin sections with hematoxylin and eosin. Five microns deparaffinized lung sections were immunostained with CD5 monoclonal CYP1A1/2 antibody (generous gift from Dr. Paul E. Thomas; dilution 1:50) for CYP1A1/2 and rat anti-mouse neutrophil antibody (Serotec, Raleigh, NC; MCA771G, dilution 1:200) for neutrophils, followed by staining with biotinylated secondary antibodies (Vector Laboratories Burlingame, CA). To analyze the degree of pulmonary neutrophil infiltration, the positively stained cells were counted in 20 non-adjacent areas per mouse under 40× magnification. A pulmonary pathologist, who was blinded to the treatment of mice with various regimens, evaluated the histopathology and immunohistochemistry slides. Assessment of lung injury in the histopathological lung sections was performed as follows: 20 random high-power fields (400× total magnification) were independently scored in a blinded fashion taking care that 50% of each field was occupied by lung alveoli. Five histological findings: neutrophils in the alveolar space, neutrophils in the interstitial space, hyaline membranes, proteinaceous debris filling the airspaces and alveolar septal thickening were graded using a three tiered schema as described in the official American thoracic society workshop report on measurement of acute lung injury in experimental animals (Matute-Bello et al., 2011) resulting in a lung injury score between zero and one.

*TUNEL analysis.* Lung tissue was analyzed for terminal deoxynucleotidayl transferase dUTP-mediated nick-end labeling (TUNEL) using the Millipore Apoptag Peroxidase In Situ apoptosis detection kit (Millipore, S7100). Tissue sections were deparaffinized and rehydrated in an ethanol series. The tissue sections were then pretreated with proteinase K for 15 min at room temp. Endogenous peroxide was quenched using 3% hydrogen peroxide in PBS for 5 min at room temp. Nicked DNA ends were labeled by the terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL) method following the protocol provided by the manufacturer. Color was developed in peroxidase substrate, counter stained with methyl green. The sections were then dehydrated and permanently mounted. Twenty consecutive hpf under 40× magnification were observed for the number of TUNEL positive cells. Negative controls (performed simultaneously but without TdT) were examined at the same time.

*Lung $F_2$-isoprostane analyses.* Mouse tissues (lung) were prepared for LC–MS/MS analysis by homogenizing the tissues in dPBS (Dulbecco's Phosphate Buffered Saline) or methanol using MP Biomedical lysing matrix D in an MP FastPrep 24 instrument (MP Biomedical, Solon, OH). The tissues were homogenized at a tissue/volume (w/v) concentration of 50 mg tissue per ml of methanol. Complete homogenization required 40 s duration at a power setting of 6. Lung tissues were suspended in PBS. Following homogenization, the lung tissue homogenates were vortex mixed and 200 µl of the homogenates was removed and placed into a 15 ml glass tube, 22 µl of 1 µg/ml deuterated 8-isoprostane $F_2$-α (8-iso-PGF2α) was added as an internal standard and the solution was then extracted twice with 3 ml of methyl tertiary butyl ether. The extracts were combined and evaporated to dryness with nitrogen gas. The dried extract was reconstituted to 100 µl volume with 50:50 methanol:0.2% acetic acid in water and transferred to an LC–MS/MS vial for analysis.

The detection and quantification of 8-iso-PGF2α were done using a Waters QuattroUltima mass spectrometer (Waters, Milford, MA) coupled to an Agilent 1100 binary HPLC system (Agilent, Santa Clara, CA). A Phenomenex Luna phenyl-hexyl 150 × 2.1 mm, 3 µ-particle size analytical column (Phenomenex, Torrance, CA) was used to chromatographically resolve 8-iso-PGF2α from background. Chromatography was done using a linear methanol:0.2% formic acid in water gradient. The initial conditions were 50% methanol to 80%