Hypoxic Stress Exacerbates Hyperoxia-Induced Lung Injury in a Neonatal Mouse Model of Bronchopulmonary Dysplasia

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Key Words

Bronchopulmonary dysplasia • Hypoxia • Hyperoxia • Lung injury • Oxidative stress • Glutathione • Protein carbonyls

Abstract

Background: Premature infants with lung injury often experience intermittent episodes of hypoxemia. **Objective:** This study investigates whether intermittent hypoxemia exacerbates oxidative stress and lung injury in neonatal mice in a hyperoxia-induced model of bronchopulmonary dysplasia (BPD). **Methods:** For the BPD model, 3-day-old C57Bl/6J mice were exposed to hyperoxia (65% O₂) for 4 weeks (O₂ group) or to hyperoxia and intermittent (10 min daily) hypoxia (O₂ + H group). Upon completion of O₂ or O₂ + H exposure, the degree of pulmonary alveolarization and granulocytic infiltration were examined. The severity of oxidative injury in lungs was defined by tissue glutathione and protein carbonyl content. Data were compared to those in naïve mice and mice subjected only to intermittent hypoxia. **Results:** Hyperoxia-exposed mice exhibited a dramatic ($p < 0.0001$) decrease of alveolarization, significantly increased granulocytic infiltration ($p < 0.0001$) and increased protein carbonyl content ($p = 0.04$) compared to naïve mice. However, O₂ + H mice demonstrated significantly ($p = 0.03$) fewer alveoli compared

to their O₂ counterparts. This was associated with a significantly ($p = 0.02$) decreased pulmonary total/oxidized glutathione ratio and a significant ($p = 0.03$) elevation of protein carbonyl content. **Conclusions:** Thus, intermittent hypoxic stress during hyperoxic induction of BPD in mice potentiates oxidative stress in lung tissue and exacerbates alveolar developmental arrest.

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Introduction

Bronchopulmonary dysplasia (BPD) is a leading cause of morbidity and mortality among premature neonates [1]. Over the past 2 decades, the histological presentation of BPD has changed from heterogeneous pulmonary inflammation and fibrosis to uniform arrest of alveolar development [2]. Oxidative stress induced by exposure to supraphysiological concentrations of oxygen (O₂) is one of the proposed mechanisms of alveolar developmental arrest in BPD [3–5]. It has been shown that exposure to hyperoxia or hypoxia results in increased accumulation of reactive oxygen species (ROS) in lung tissue [6, 7]. To prevent oxidative injury, organisms have evolved different antioxidant systems (superoxide dismutase, catalase,

glutathione peroxidase and various thio-, peroxi- and glutaredoxins). Glutathione is viewed as the key component of all antioxidant defenses [8].

Premature infants are known to experience intermittent episodes of hypoxemia lasting from a few seconds to several minutes [9]. Furthermore, infants who develop BPD experience more frequent episodes of O₂ desaturations than infants who recover from respiratory distress syndrome without developing BPD [10, 11]. Given that intermittent hypoxia has been shown to exert oxidative stress [12, 13], we hypothesized that intermittent hypoxemia exacerbates oxidative stress, which results in more severe lung injury in mice with hyperoxia-induced BPD.

Materials and Methods

The BPD Model and Study Design

BPD in neonatal mice was produced as we described previously [14]. Three-day-old (p3) C57Bl/6J mice of both genders were purchased from Jackson Laboratories (Bar Harbor, Me., USA). p3 mice are at the saccular stage of their lung development, which corresponds to lung development in the human fetus at 28–30 weeks of gestation [15]. Pups from different litters were randomly distributed between dams (6 pups per dam) to minimize a litter-dependent bias. Body weights prior to and after completion of BPD modeling were recorded. The experimental protocol was approved by the Columbia University Institutional Animal Care and Use Committee. Briefly, p3 mice were exposed to 65% O₂ for 4 weeks by constant delivery (0.5 liters/min) of 100% O₂ into a custom-made 1.2-m³ plastic chamber to maintain the fraction of inspired oxygen at 0.65 ± 0.03 . The O₂ concentration inside the chamber was constantly monitored. Hyperoxia exposure was interrupted for 10 min daily to clean the cages and exchange dams between experimental and naïve (not exposed to O₂) mice in order to minimize any adverse effect of hyperoxia on milk production by the dams.

To mimic desaturation episodes in premature infants requiring O₂ therapy, randomly selected mice were exposed to brief (10 min) intermittent hypoxic stress during the course of hyperoxia. The hypoxic stress consisted of exposure to 8% O₂ (N₂ balanced) daily for 1 week and every other day for another week. Each mouse was exposed to the total of 10 hypoxic episodes. The timing for hypoxic exposure (first 2 weeks of life) was chosen because mouse lungs go through the saccular and early alveolar stages of development during the first 2–3 postnatal weeks [15]. Given that the pathological hallmark of BPD in human neonates is alveolar developmental arrest, hypoxic challenges were applied during this vulnerable period of alveolar formation. During the hypoxic challenge, the O₂ saturation and heart rate were monitored in randomly selected mice using pulse oximetry (Nonin Medical Inc., Plymouth, Minn., USA). The duration of hypoxic episodes was limited to 10 min, because during this period of time, animals demonstrated O₂ desaturation (oxygen saturation = $60 \pm 3.26\%$) without bradycardia.

The following experimental groups were studied (fig. 1a):

- (1) Hyperoxia (O₂) group (n = 10): mice were exposed to hyperoxia alone without hypoxic challenge. This group was designed to model BPD.
- (2) Hyperoxia + hypoxia (O₂ + H) group (n = 15): mice were exposed to 65% O₂ for 4 weeks and subjected to hypoxic stress during the first 2 weeks of hyperoxia. This group was designed to mimic infants who experience brief episodes of desaturations at the initial stage of BPD development.
- (3) Normoxia + hypoxia (NH) group (n = 10): mice were subjected to intermittent hypoxic stress during the first 2 weeks of life. This group was designed to discriminate the impact of intermittent hypoxia from that of combined hyperoxic/hypoxic stress on the alveolar development.

All data obtained from experimental groups were compared to those of age-matched normoxic naïve mice (n = 5) that were not exposed to hyperoxia or hypoxia.

Upon completion of BPD modeling, the severity of lung injury was defined by histopathological evidence of alveolar developmental arrest.

Pulmonary Histopathology

At 4 weeks of age, anesthetized mice were sacrificed by thoracotomy and transection of the thoracic aorta. Lungs were perfused with phosphate buffer solution with EDTA. Following perfusion, lungs were filled via the trachea with the same buffer solution under pressure of 20 cm H₂O for 5 min. The right lung was snap frozen in liquid nitrogen and processed for measurement of glutathione and protein carbonyl content. The left lung was fixed in 4% paraformaldehyde and embedded in paraffin. Five-micrometer sections (100 μ m apart) were obtained from the anterior, middle and posterior frontal planes and stained with hematoxylin and eosin.

Radial Alveolar Count. The degree of alveolarization was defined by the radial alveolar count (RAC) [16, 17]. Briefly, an investigator 'blinded' to the study groups performed RACs during examination of digital images captured under identical magnification. The RAC was the number of distal air sacs that were transected by the line drawn perpendicular from the most proximal to the pleura terminal bronchiole toward the nearest pleura. Three RACs were obtained from each section (9 RACs for each mouse). The mean value of all RACs for each mouse was used for statistical analysis.

Immunohistochemistry. Detection of Ly6G/C antigen-positive cells (granulocytes) in lung tissue was used as a marker of inflammatory cell infiltration. Initially, 5- μ m sections were blocked with appropriate secondary antibody serum. Then, specimens were incubated overnight at 4°C with primary rat antimouse Ly6G/C monoclonal antibodies (BD Bioscience, San Jose, Calif., USA). Following incubation with secondary goat antirat Alexa Fluor-conjugated antibodies (Invitrogen, Carlsbad, Calif., USA), sections were examined using a Bio-Rad 2000 confocal microscope. To analyze the degree of pulmonary granulocytic infiltration, Ly6G/C-positive cells were 'blindly' counted in 9 nonadjacent areas [3 areas per section, 3 sections (anterior, middle and posterior) per mouse] captured under identical magnification and fluorescence. The mean number of Ly6G/C-immunopositive cells per square millimeter of pulmonary tissue for each animal was used for data analysis.

Markers of Oxidative Stress and Injury in Lungs

The oxidative lung injury was defined as follows: (1) as an increase in the tissue content of products of molecular oxidative damage, i.e. protein carbonyls (products of protein oxidation), coupled with (2) assessment of the tissue antioxidative capacity, i.e. total/oxidized glutathione ratio. Briefly, following homogenization, lung tissue samples were centrifuged for 2 min at 10,000 g. The supernatant was used for the measurement of pulmonary total and oxidized glutathione and protein carbonyl content. Total protein concentration in the lung tissue was determined according to the method of Bradford [18].

Measurement of Protein Carbonyls. Protein carbonyl content in the lung tissue was determined spectrophotometrically at 340 nm using a protein carbonyl assay kit (Cayman Chemical Co., Ann Arbor, Mich., USA). The protein carbonyl concentration was expressed as nanomoles per gram of total protein.

Measurement of Glutathione. Total (reduced plus oxidized) and oxidized tissue glutathione concentrations were measured as described by Griffith [19] with minor modifications. Briefly, to prevent self-oxidation, tissue samples were diluted in 5% metaphosphoric acid (1:1). Three working solutions were used: (1) 0.3 mM NADPH (Sigma-Aldrich, St. Louis, Mo., USA), (2) 6 mM 5,5'-dithiobis-2-nitrobenzoic acid (Sigma-Aldrich) and (3) 10 U/m glutathione reductase (Sigma-Aldrich). Reduced glutathione at concentrations of 0 to 20 μ M was used as standard (Sigma-Aldrich). The total glutathione concentration was measured at 405 nm using a microplate reader (Tecan, Männedorf, Switzerland). To measure the concentration of oxidized glutathione, samples were first normalized with 4 N NaOH to pH 5.5–7.0. Then, the fraction of reduced glutathione in the sample was quenched using 2-vinylpyridine (Sigma-Aldrich), followed by the measurement of total glutathione as described above. Total and oxidized glutathione tissue contents were expressed in micromoles per gram of total protein. The ratio of total to oxidized glutathione was calculated and used as a measure of antioxidant capacity.

Statistical Analysis

Data are expressed as means $\pm$ SEM. Student's *t* test or Mann-Whitney analysis (depending on distribution of the data) were used to compare results between O₂ and O₂ + H mice, between naïve and NH mice and between O₂ and naïve groups. Values were considered significantly different if the *p* value was ≤ 0.05 .

Results

Outcome of Hyperoxia-Induced BPD

Weight. All mice had similar body weight at the start of the experiment (data not shown). Hyperoxic exposure resulted in a significantly decreased weight at the end of BPD modeling compared to naïve littermates (O₂ mice 6.22 ± 0.3 g vs. naïve mice 10.6 ± 1.3 g; *p* = 0.004). Intermittent hypoxic stress in O₂ + H mice did not lead to a significant reduction in body weight gain (5.9 ± 0.8 g; *p* = 0.6) compared to their O₂ counterparts. Normoxic mice exposed to intermittent hypoxic stress also demon-

strated significantly decreased body weight (NH mice 7.6 ± 0.3 g; *p* = 0.04) compared to their naïve littermates.

Pulmonary Histopathology. Mice exposed to 65% O₂ for 4 weeks exhibited robust histological signs of alveolar developmental arrest, i.e. poor alveolar septation and enlarged terminal air sacs (fig. 1b, c). These hyperoxia-exposed mice demonstrated a significantly (*p* < 0.0001) decreased RAC compared to that in normoxic animals (fig. 1b). However, O₂ + H mice subjected to intermittent hypoxic stress during hyperoxic exposure exhibited the lowest RAC, suggesting that hypoxic stress exacerbates the alveolar underdevelopment induced by hyperoxic exposure (fig. 1b). There was no difference in the degree of alveolarization between NH mice (RAC = 12.02 ± 0.54) and naïve mice (RAC = 12.69 ± 0.44 ; fig. 1b).

Inflammatory Changes in Lungs

Immunohistochemical staining for Ly6G/C revealed significantly (*p* < 0.0001) increased numbers of granulocytes in the lungs of O₂ animals compared to those in naïve mice (fig. 2a). Intermittent hypoxic exposure in normoxic mice also resulted in a significantly (*p* = 0.006) greater pulmonary granulocytic infiltration compared to that in naïve (normoxic) mice. When mice subjected to hyperoxia were compared to mice subjected to hyperoxia plus intermittent hypoxia, no difference in pulmonary granulocytic infiltration was detected (fig. 2a).

Oxidative Lung Injury

Mice exposed to isolated hyperoxia demonstrated significantly (*p* = 0.04) elevated protein carbonyl content in their lung tissue compared to that in naïve mice (fig. 2b). The lung tissue level of oxidized glutathione was significantly increased in both O₂ mice and O₂ + H mice compared to naïve mice (fig. 2c). However, in O₂ mice, the elevation of oxidized glutathione content was paralleled by a significant elevation of the total glutathione level in lung tissue (33.4 ± 3.5 μ mol/g protein; *p* = 0.04) compared to naïve mice (22.7 ± 3.3 μ mol/g protein). As a result, there was no difference in the total/oxidized pulmonary glutathione ratio (the marker of glutathione antioxidant capacity) in O₂ mice compared to their naïve counterparts (fig. 2d). In contrast, in O₂ + H mice, a significant rise in the oxidized glutathione level was not associated with a significant rise in total glutathione content in their lung tissue (data not shown). Moreover, O₂ + H mice demonstrated a significantly (*p* = 0.04) higher level of oxidized glutathione compared to O₂ animals (fig. 2c). This resulted in a significant (*p* = 0.02) reduction in the total/oxidized glutathione ratio compared to that

Fig. 1. Schema of the study design (**a**), RAC (**b**) and pulmonary histopathology (**c**) in naïve (N; n = 5), NH (n = 10), O₂ (n = 10) and O₂ + H (n = 15) mice. **b** Data are means $\pm$ SEM. ^a $p < 0.0001$ compared to O₂ mice and ^b $p < 0.0001$ compared to naïve mice. **c** Shown are 5-μm frontal lung sections stained with hematoxylin and eosin. Magnification $\times 10$.

in mice exposed only to O₂ (fig. 2d). This depletion of the glutathione antioxidant capacity in O₂ + H animals was associated with exacerbation of oxidative damage to pulmonary proteins, as the protein carbonyl content was substantially increased ($p = 0.05$) compared to O₂ mice (fig. 2b). There was no significant difference in pulmonary protein carbonyl content, total/oxidized glutathione ratio or the level of oxidized glutathione between NH and naïve mice (fig. 2b–d).

Discussion

There are 2 major findings of this study: (1) intermittent, brief hypoxic stress significantly exacerbates the degree of alveolar developmental arrest induced by hyper-

oxic exposure in neonatal mice and (2) the mechanism of this exacerbation is linked to a significant amplification of oxidative lung injury.

Oxidative injury is a well-known mechanism for the development of BPD in human and animal neonates [5, 20]. It has been reported that exposure of the lungs to high oxygen concentrations results in the exhaustion of antioxidant systems and in a robust increase in products of lipid and protein peroxidation in pulmonary tissue [21, 22]. This is associated with severe alveolar developmental arrest in neonatal rats [23]. Our study using a neonatal murine model demonstrates that chronic hyperoxia exposure results in a significant elevation of protein carbonyls and oxidized glutathione content in lung tissue associated with a profound alveolar hypoplasia. Importantly, our experimental design is the first to highlight

Fig. 2. The total number of Ly6G/C-immunopositive cells per square millimeter of pulmonary tissue (**a**), pulmonary protein carbonyl content (**b**), pulmonary oxidized glutathione content (**c**) and total/oxidized glutathione ratio (**d**) in naïve (black bars; $n = 5$), NH (gray bars; $n = 10$), O_2 (white bars; $n = 10$) and $O_2 + H$ (striped bars; $n = 15$) mice. Data are means $\pm$ SEM. **a** ^a $p = 0.006$ and ^b $p < 0.0001$ compared to naïve mice. **b** ^a $p = 0.05$ compared to O_2 mice and ^b $p = 0.04$ compared to naïve animals. **c** ^a $p = 0.01$ compared to O_2 mice and ^b $p = 0.03$ compared to their naïve littermates. **d** ^a $p = 0.04$ compared to O_2 mice.

the critical role of intermittent hypoxic stress in the exacerbation of oxidative pulmonary injury in a mouse model of BPD. Mice that were subjected to the combination of intermittent hypoxia and hyperoxia developed the most severe oxidative lung injury and the most profound alveolar developmental arrest. Although mice subjected to isolated hyperoxia exhibited a significant increase in oxidized glutathione content in their lungs, the total/oxidized glutathione ratio did not differ from that in their naïve counterparts. This can be explained by the compensatory rise in the tissue pool of total glutathione in response to the oxidative stress load induced by chronic O_2 exposure. It has been shown that the level of reduced glutathione increases in response to hyperoxia in lung tissue in rodents [24, 25] and in human alveolar epithelial cell culture [26]. Although the exact cellular mechanism for this phenomenon remains unknown, it has been suggested that the increase in gamma-glutamylcysteine synthase activity is responsible for the increase in intracellular glutathione during oxidative stress in alveolar epithelial cells [27]. As a result, the major marker of antioxidant capacity in the glutathione system (the total/oxidized glutathione ratio) remained unchanged. In contrast, mice subjected to the combination of hyperoxia and hypoxia exhibited the highest level of protein oxidation products associated with a failure to maintain a normal

level of the total/oxidized glutathione ratio. This indicates an exhaustion of their glutathione antioxidant capacity. Thus, our data suggest that the severity of oxidative stress in lungs is potentiated by intermittent hypoxic episodes during hyperoxic exposure.

It is well documented that both isolated hypoxia and isolated hyperoxia induce severe oxidative stress [28]. However, there are no reports on pulmonary oxidative injury following exposure to intermittent hypoxia-hyperoxia. Interestingly, using a neonatal rat model of retinopathy of prematurity, Penn et al. [29] demonstrated that the extent of retinal damage increased linearly with the increase in the difference in the two oxygen concentrations to which a treatment group was subjected. York et al. [30] demonstrated that very-low-birth-weight infants experiencing fluctuating levels of partial pressure of arterial oxygen were at higher risk for threshold-stage retinopathy of prematurity compared to those neonates that had relatively constant levels of partial pressure of arterial oxygen.

Clinical observation indicates that in spite of continuous oxygen therapy, premature infants experience multiple episodes of hypoxemia (O_2 desaturations) associated with apnea of prematurity, patent ductus arteriosus and infections, among other causes. Recently, it has been shown that the treatment of apnea of prematurity with

caffeine was strongly associated with a significant reduction in the incidence of BPD [31]. Moreover, several investigators have reported that infants who develop BPD experience more frequent episodes of O₂ desaturations than those infants who recover from respiratory distress syndrome without developing BPD [10, 11]. Our experimental results suggest that the mechanistic link between frequent episodes of hypoxemia and the development of BPD observed in human neonates is exacerbation of oxidative lung injury triggered by the fluctuation in the fraction of inspired oxygen and oxygenation.

In our study, we did not determine the source of ROS. Although activated inflammatory cells (granulocytes) infiltrating lungs in BPD have been shown to produce ROS [32], our results indicate that it is unlikely that granulocytes are responsible for the exacerbation of oxidative injury in O₂ + H mice. The degree of pulmonary granulocyte infiltration in O₂ + H mice was similar to that in mice exposed to hyperoxia alone. In addition, significantly increased pulmonary infiltration with granulocytes in NH mice was not associated with the induction of oxidative lung injury. The potential source of ROS in our experiments might be pulmonary mitochondria. Mitochondria are known to be the major site for the production of ROS in intact and stressed (postschismic, hyperoxia-exposed) tissue. It has been shown that following

hyperoxic exposure, pulmonary endothelial cells significantly increase production of ROS originating from the mitochondrial electron chain [33, 34], and that glutathione reductase gene transfer to mitochondria significantly protected H441 cells against hyperoxia-induced growth arrest and improved the glutathione/oxidized glutathione ratio in hyperoxic cells [35]. Given that glutathione reductase is a key enzyme supporting intramitochondrial glutathione antioxidant capacity, these data suggest that the ROS of mitochondrial origin are involved in cellular growth arrest during hyperoxia [36].

In conclusion, our data demonstrate that intermittent hypoxic stress during hyperoxic induction of BPD in neonatal mice significantly exacerbates oxidative lung injury and alveolar developmental arrest. These results suggest that the aggressive prevention of hypoxic episodes in neonates at risk for the development of BPD should be considered a therapeutic target to attenuate the extent of oxidative lung injury.

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