Prolonged Moderate Hyperoxia Induces Hyperresponsiveness and Airway Inflammation in Newborn Rats

DELPHINE DENIS, MICHAEL JOHN FAYON, PATRICK BERGER, MATHIEU MOLIMARD,
MANUEL TUNON DE LARA, ETIENNE ROUX, AND ROGER MARTHAN

Pediatric Intensive Care and Pulmonology Units, Hôpital Pellegrin-Enfants, Place Amélie Raba Léon, 33076 Bordeaux Cedex, France [D.D., M.J.F.]; and Laboratoire de physiologie cellulaire respiratoire, INSERM E-9937 - Université Victor Segalen Bordeaux 2, 146 rue Léo Saignat, 33076 Bordeaux Cedex, France [D.D., M.J.F., P.B., M.M., M.T.D.L., E.R., R.M.]

ABSTRACT

Bronchopulmonary dysplasia is the most common cause of chronic pulmonary disease in premature infants. Airway inflammation appears to play a major pathogenetic role together with barotrauma and oxygen toxicity. The aim of the present study was to determine the effect of a 15-d exposure to moderate hyperoxia (Fio_2 , 50%) on airway reactivity and inflammatory response in neonatal and adult rats. We studied in isolated tracheal rings the 1) isometric contraction to cumulative concentrations of carbachol (10^{-8} to 10^{-3} M); 2) epithelial, submucosal, smooth muscle, and connective tissue surface area; and 3) distribution of inflammatory cells (mastocytes, granulocytes, macrophages) by using MAb. Reactivity to carbachol was significantly increased in the hyperoxic pups, in which a 13% increase in tracheal smooth muscle surface area was observed. Type-I mast cells and macrophages (submucosa and connective tissue) and granulocytes (connective tissue) were increased in the

neonatal hyperoxic group. Hyperoxia did not influence functional, morphometric, or cellular data in adult rats. In conclusion, exposure of newborn rats to moderate hyperoxia induces airway hyperresponsiveness and histologic changes similar to those reported in bronchopulmonary dysplasia. Hyperresponsiveness may be ascribed to an increase in smooth muscle related to the release of yet undetermined mediators by inflammatory cells infiltrating the airways. Lung immaturity definitely plays a role because similar alterations are not observed in adult rats. (*Pediatr Res* 50: 515–519, 2001)

Abbreviations

BPD, bronchopulmonary dysplasia

Fio_2 , fraction of inspired oxygen

O_2 , oxygen

RMCP-I and II, rabbit anti-rat mast cell protease I and II

BPD, the most common chronic obstructive pulmonary disease in children, develops mostly in premature infants who require prolonged ventilation and/or O_2 therapy (1). Airway hyperreactivity, which can persist into adolescence, is recognized as one of the long-term sequelae of this condition.

Despite improved neonatal care, including surfactant therapy and new ventilation modalities such as high-frequency oscillation, the incidence of BPD is increasing. The precise etiology of the disease remains unclear. Barotrauma related to ventilation induces airway remodeling and hyperreactivity by overdistension of lung tissue (2). However, hyperoxia, which causes more significant physiologic, inflammatory, and histologic changes than barotrauma alone, may play a critical role in

the etiology of airway hyperreactivity related to BPD (3). Similar to asthma, pulmonary inflammation is believed to play an important role in the pathogenesis of chronic lung disease. Histologic features of infants dying from BPD include submucosal edema, chronic inflammation, squamous metaplasia of epithelial cells in large and small airways, thickening of airway smooth muscle, and peribronchiolar fibrosis (4).

In the past, various hyperoxic models have been studied. In most instances, high Fio_2 (>90%) for short exposure times (<8 d) has been used (5, 6). Such protocols have induced morphologic (airway smooth muscle thickening) as well as functional (increased airway hyperresponsiveness) changes in young 21-d-old rats (7). However, such protocols did not address the specific issue of lung immaturity because similar effects were observed in the adult rat (7). Moreover, such conditions of exposure to hyperoxia do not correspond to the present-day realities in an intensive care unit. Premature babies usually benefit from much lower levels of Fio_2 for a longer period of time.

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Correspondence: Michael Fayon, M.D., M.Sc., Service de Réanimation Pédiatrique, Hôpital Pellegrin-Enfants, Place Amélie Raba Léon, 33076 Bordeaux Cedex, France; e-mail: michael.fayon@chu-bordeaux.fr

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The aim of the present study was to assess the combined effect of immaturity and realistic (*i.e.* moderate but prolonged) hyperoxia on airways. We have established an animal model of BPD induced by prolonged but moderate hyperoxia in the newborn rat. We have examined the impact of such exposure on airway reactivity, morphology, and inflammation and have observed that, under these conditions, hyperoxia-induced alterations were restricted to newborn rats.

METHODS

Exposure to hyperoxia. All animals were housed in our animal laboratory, which is approved by our Institutional Board. For each series of experiments, pregnant pathogen-free Wistar rats of known gestational age were purchased. After parturition in the laboratory, the pups were redistributed at random between the mothers. Within 24 h of birth, the pups were either exposed to moderate hyperoxia (50% O₂) or normoxia (21% O₂) at ambient pressure (sea level) for 15 d in standard cages placed within 86-L capacity Plexiglas isolation chambers (Roller 6 Rothos Sundis, France). This level of exposure, which is in contrast to most previous studies (Fio₂ ≥ 95% for 8 d) (5, 8), was chosen to take into account the change in the way O₂ is now given to neonates in clinical practice (9). They were housed with one nursing mother during the entire exposure time.

In the hyperoxic group, the O₂ fraction in the closed-circuit chambers was maintained at a constant value of 50 ± 1% by continuous monitoring using an oxygen analyzer (Servomex O₂ Analyzer 580A, England) that controlled an electric valve at the output of the O₂ tank. Gas circulation was facilitated by an air pump (3.5 L/min). In the control group, rats were housed in a similar chamber, and ambient air was continuously flushed through the control chambers via an identical air pump. Mothers were given food and water *ad libitum*. Cages were removed from the isolation chambers for 5 min every day to ensure servicing. Moisture and CO₂ were absorbed with silica gel and soda lime (Prolabo, France), respectively, and both were changed every day. Pairs of adult rats were also exposed either to hyperoxia or normoxia for 15 d under the same conditions as rat pups.

Isometric contraction measurement. On d 15, the animals were removed from their chambers and anesthetized by intraperitoneal administration of ethyl carbamate (pups, 80 mg; adult rats, 1 g). The body weight of each pup was then recorded. The trachea was rapidly excised, discarded of its connective tissue, and immediately placed in Krebs-Henseleit (KH) solution (NaCl, 118.4 mM; KCl, 4.7 mM; CaCl₂·2H₂O, 2.5 mM; MgSO₄·7H₂O, 1.2 mM; KH₂PO₄, 1.2 mM; NaHCO₃, 25 mM; D-glucose, 11.1 mM).

Each pup trachea was studied as a single segment due to its small size. Each trachea was resected into a 7-mm long ring so as to decrease variability in force generated as a result of unequal longitudinal smooth muscle content. In contrast, adult tracheas were cut into four rings of similar length (4 mm). They were mounted in an isolated organ bath system filled with KH solution, bubbled with 95% O₂-5% CO₂, and maintained at 37°C as previously described (10). After a 60-min equilibration

period, each preparation was preloaded at the optimal resting tension (L₀ = tension that allows the maximal reproducible active force generation) (11). L₀ was determined by generating a resting tension-force generation curve using acetylcholine (10⁻³ M) (Sigma Chemical Co., Saint-Quentin-Fallavier, France) in both normoxic and hyperoxic pups. L₀ corresponded to a passive load of 1750 mg in normoxic pups and 2000 mg in hyperoxic pups; L₀ for adult rats was found to be at 2000 mg in both groups. A cumulative concentration-response curve to carbachol from 10⁻⁸ to 10⁻³ M (Sigma Chemical Co., Saint-Quentin-Fallavier, France) was then constructed. For each ring, the contractile response to carbachol was expressed both as active force (in mg), *i.e.* the total force minus the resting tension, and as stress (g/mm²), *i.e.* the active force divided by muscle cross-sectional area.

E_{max}, which depicts the maximal active force generated (final plateau on the cumulative concentration-response curve), and EC₅₀, the concentration of agonist producing half-maximal response, were calculated for each curve. EC₅₀ was calculated by nonlinear curve fitting using a logistic function and expressed as log EC₅₀.

Morphologic study. Subsequent to the organ bath study, both control tissues and tissues from hyperoxic animals were embedded in glycolmethacrylate (GMA) (see below) and cut into 2-μm thick sections perpendicular to the long axis. For the morphometric study, sections were stained with toluidin blue. Light microscopy was performed using an Optiphot microscope (Nikon, Tokyo, Japan). A compartmental analysis was performed delineating epithelium, conjunctive tissue, submucosa, and smooth muscle layer. The area (μm²) and epithelium thickness (μm) (layer area divided by the length of the epithelial basement membrane) were calculated using a video interactive display system (×40 magnification) and an appropriate software (Quancoul, Quant'Image 1995-7, France) as previously described (12). Mean tracheal smooth muscle cross-sectional area (n) was determined from the data obtained in three nonadjacent sections of the same trachea performed perpendicularly to the long axis of the specimen.

Sample processing for immunohistochemistry. The tracheal specimens embedded in GMA were also used for immunostaining as described previously (12, 13). The GMA sections were cut at 2-μm thickness perpendicular to the long axis with an ultramicrotome and incubated overnight at room temperature with mouse or rabbit antibodies (Ab) including RMCP-I and mouse II anti-rat mast cell protease (Moredum, Edinburgh, UK), mouse anti-rat macrophages (Serotec, Oxford, UK), and mouse anti-rat granulocytes (Serotec, Oxford, UK). Control slides were treated similarly, applying an irrelevant antibody.

Quantification of immunostaining. Light microscopy was performed using an Optiphot microscope (Nikon, Tokyo, Japan). Cells staining positively with each antibody were counted at the magnification of ×200 in epithelium, submucosa, smooth muscle, and connective tissue. Cell counts were expressed as density, *i.e.* number of cells/mm² of tissue.

Statistical and data analysis. All data are reported as mean ± SEM except for cell densities, which are expressed as median values. Statistical analysis was performed using the software package NCSS 6.0.21 (Kaysville, UT, U.S.A.). The

effect of hyperoxic exposure on somatic growth, epithelial thickness, smooth muscle, submucosa, and connective tissue area was assessed by unpaired *t* test. Two-way ANOVA for repeated measures was used to analyze the effect of hyperoxia on force generation and to determine whether the two curves were different from each other. Differences identified by ANOVA were pinpointed using the Newman-Keuls multiple-range test for individual comparison. Results from immunohistologic studies were analyzed using the Mann-Whitney unpaired test to compare the number of inflammatory cells exposed to hyperoxia *versus* normoxia. Results were considered significant at $p < 0.05$.

RESULTS

Neonatal Rats

Effect of hyperoxia on survival and somatic growth. All animals survived. Hyperoxic exposure significantly reduced somatic growth; final weight was 26.9 ± 0.4 g in the air-exposed group ($n = 59$) *versus* 21.6 ± 0.3 g in the O_2 -exposed group ($n = 59$; $p < 0.001$).

Effect of hyperoxia on tracheal growth and morphometry. Hyperoxia significantly increased the smooth muscle area: $39,000 \pm 1,400 \times \mu m^2$ (air-exposed, n animals = 89) *versus* $44,000 \pm 1,800 \mu m^2$ (O_2 -exposed, n animals = 89); $p = 0.04$. No effect of hyperoxia on the submucosal, connective tissue, or epithelial area was observed. Total tracheal tissue area was not significantly modified in the O_2 -exposed group ($68 \times 10^4 \pm 2 \times 10^4 \mu m^2$, $n = 37$) *versus* the air-exposed group ($72 \times 10^4 \pm 2 \times 10^4 \mu m^2$, $n = 38$); $p = 0.23$.

Effect of hyperoxia on rat isolated tracheal ring contractility. The mean maximal isometric tension generated in response to carbachol was greater in the trachea from hyperoxia-exposed pups compared with controls (Fig. 1A). However, there was no difference in terms of sensitivity as indicated by EC_{50} : -6.82 ± 0.28 log M (air-exposed) *versus* -6.36 ± 0.16 log M (O_2 -exposed); $p > 0.05$.

After normalization to the smooth muscle cross-sectional area, the concentration-stress curve showed no difference in airway reactivity between the two groups (Fig. 1B). Sensitivity to carbachol also remained unchanged.

Effect of hyperoxia on inflammatory cell infiltration in rat trachea. Light microscopy revealed no alteration in tracheal tissue morphology. Overall inflammatory cell density was significantly increased in the hyperoxic connective tissue only (mean, 24.31 ± 39.33 cells/mm³ in the hyperoxic group *versus* 5.99 ± 14.75 cells/mm³ in the normoxic group; median, 0 in both groups); $p = 0.01$. A significant increase in macrophages was noted in the hyperoxic group in the whole tracheal section (Fig. 2A). When each histologic layer was studied separately, connective tissue-type mast cell (RMCP-I) and macrophage densities were found significantly higher in both submucosa and connective tissue in O_2 -exposed specimens compared with control (Fig. 2B) (RMCP-I in connective tissue, 18.40 ± 6.83 *versus* 56.64 ± 11.4 in the normoxic and hyperoxic groups, respectively). Density of granulocytes was also increased in this latter tissue (Fig. 2B). No mucosal-type mast cell marker (RMCP-II) was detected in either group.

Figure 1. Effects of exposure to O_2 (F_{iO_2} , 50%) during 15 d on the cumulative concentration-response curve (CCRC) to carbachol in newborn rat isolated tracheal rings. Filled symbols indicate mean values of contractile force in the O_2 -exposed group ($n = 8$). Open symbols indicate mean values for the air-exposed group control ($n = 8$). Vertical bars indicate SEM. (** $p < 0.01$). (A) Contractile force expressed as active force (mg). (B) Contractile force corrected for cross-sectional area (stress, g/mm²).

Adult Rats

In contrast with newborn rats, we did not observe any significant difference between the two groups in terms of smooth muscle area, $69,000 \pm 440 \mu m^2$ (air-exposed, $n = 45$) *versus* $75,000 \pm 40$ (O_2 -exposed, $n = 37$), or in terms of submucosal, connective tissue, or epithelial surface area (data not shown).

No difference was noted in airway contractile response to carbachol expressed either in terms of active force [E_{max} , 2510

Figure 2. Cell phenotypes detected by MAb in GMA-embedded air-exposed specimens (white box) or O₂-exposed specimens (striped box). Antibodies used were anti-connective tissue rat mast cells (RMCP-I), anti-mucosal rat mast cells (RMCP-II), anti-macrophages, and anti-granulocytes. Inflammatory cell numbers were determined in the whole tracheal section (air-exposed, $n = 45$; O₂-exposed, $n = 40$) (A) or according to cell type and histologic layer: anti-connective tissue rat mast cells ($n = 12$ per group), anti-macrophages (air-exposed, $n = 12$; O₂-exposed, $n = 8$), and anti-granulocytes ($n = 10$ per group) (B). Box and whisker plots: vertical lines represent the 5th and 95th centile points, and circles represent the range. *SbM* indicates submucosa; *CT*, connective tissue; * $p < 0.05$.

± 314 mg (air-exposed) versus 2707 ± 242 mg (O₂-exposed)] or in terms of stress [$E_{\max}$, 42.10 ± 5.93 g/mm² (air-exposed) versus 38.53 ± 7.43 g/mm² (O₂-exposed)]. Similarly, sensitivity was not modified by O₂ exposure [EC_{50} , -7.12 ± 0.67 log M (air-exposed) versus -6.24 ± 0.06 log M (O₂-exposed)]; $p = 0.21$.

There was no evidence of increased airway inflammation after hyperoxia, because there was no difference between the two groups in the distribution of inflammatory cells either in the whole tracheal section or in the different histologic layers.

DISCUSSION

The present study indicates that a moderate and prolonged hyperoxia in neonatal rats induces tracheal smooth muscle thickening leading to enhanced contractility. Hyperoxia also stimulates a cellular inflammatory reaction in the submucosa and connective tissue. The deleterious effects of such O₂ exposure are restricted to newborn rats because neither functional nor histologic alterations were observed in the adult rats.

In the present study, the increased contractility observed in the hyperoxic rat pups compared with the normoxic group is in agreement with previous studies reporting that hyperoxia at a much higher level causes pulmonary dysfunction in many animals. For example, it increases maximal airway contractility in newborn guinea pigs (14) and immature rats with increased smooth muscle (15). In premature infants, artificial ventilation results in a greater increase in smooth muscle occurring as soon as 6 d after birth (16).

We did not address the question of whether hypertrophy or hyperplasia is responsible for smooth muscle thickening. Because the increased density of inflammatory cells was only observed in the submucosa and connective tissue, it is highly unlikely that muscle thickening was due to cellular infiltration. A hyperplastic process resulting in airway epithelial and smooth muscle layer thickening induced by high O₂ exposure in immature rats has been demonstrated by cellular DNA synthesis (17). Furthermore, the lungs of O₂-exposed rats showed excess airway smooth muscle mitogenic activity, attributable to the presence of one or more non-PDGF (platelet-derived growth factor) polypeptide growth factor(s) (18). It is possible that this abnormal mitogenic activity is essential to O₂-induced airway smooth muscle remodeling observed in immature rats *in vivo*.

Similar to what we observed, airway remodeling has previously been described in BPD (1) and asthma (19). The question that arises is whether airway remodeling accounts for hyperoxia-induced airway hyperresponsiveness. In the present study, normalizing tension by the smooth muscle area abolished the difference in the reactivity for carbachol of the airways between the two groups of pup rats. This indicates that smooth muscle remodeling induced by hyperoxia contributes, at least, to the increase in reactivity of the airways. Hersenson *et al.* (5) have also demonstrated that there is a good correlation between airway hyperresponsiveness and the magnitude of airway layer remodeling. All of the above support the view that airway remodeling greatly influences airway responsiveness in these animals (20).

The following issue deals with the mechanism responsible for the airway remodeling that accompanies hyperoxia in the neonatal rat. The inflammatory reaction observed in the present model, as in BPD, is characterized by the presence of inflammatory cells able to release various inflammatory mediators including proteases, chemoattractants, cytokines, and leukotrienes (21). Interestingly, no increase in epithelial surface area or inflammatory cell recruitment was noted in our study, indicating that it is more likely that the changes were due to tissue rather than purely epithelial hyperoxia. The increase in mastocytes and macrophages in the connective tissue and submucosa as well as in granulocytes in the connective tissue may indirectly contribute to airway remodeling by releasing mitogenic factor(s). Interestingly, Lyle *et al.* (22) have demonstrated tryptase-positive mast cell hyperplasia in BPD. It is thus possible that the protease revealed by RMCP-I has mitogenic properties that may be partially or totally responsible for muscle thickening. Of note, mucosal-type mastocytes revealed by anti-RMCP-II antibodies do not appear until the age of 3 wk, which is why they were undetectable in our neonatal

model (23). Tachykinins, substance P, and both neurokinin A and B have also been implicated in the mediation of neurogenic inflammation of the airways (24–26). Several lines of evidence indicate that hyperoxia may increase tachykinin synthesis in newborn airways, leading to airway remodeling (8, 26). Whether a similar phenomenon occurs in our model deserves further studies.

The absence of deleterious effects of moderate hyperoxia in adults suggests that 1) such mechanisms were not involved in the present study and 2) rat pups are more prone to oxygen toxicity, probably due to the immaturity of their protective systems (antiproteases, antioxidants, surfactant system) and/or defective immune response (21).

In the present study, a fixed duration of exposure to O₂ of 15 d was chosen *a priori* because it has practical clinical implications. Because each animal has its own individual susceptibility to inhaled toxic substances (27), this could explain the scatter observed in the inflammatory cell counts. The variability may also have been enhanced by the young age of the animals, which are still in the process of airway maturation. However, we had no objective signs to determine when to cease exposure to have a uniform set of subjects (27).

CONCLUSION

To the best of our knowledge, this is the first study demonstrating that in rat neonates, a moderate hyperoxia (Fio₂ = 50%) for a relatively long period induces functional, morphometric, and inflammatory alterations similar to those observed in human BPD. Our findings suggest that exposure to O₂ induces airway smooth muscle remodeling responsible for airway hyperreactivity. Cellular inflammation appears to participate in this airway remodeling, presumably through the release of mitogenic factors. But the mechanisms that account for a change in function resulting from hyperoxia are only speculative and not defined in this study. Further studies are required to identify the mitogenic factor(s) involved. We believe that this model may prove useful to study the mechanisms of smooth muscle remodeling and to test therapeutic interventions relevant to the treatment of BPD.

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