Structural and Functional Abnormalities of the Airways of Hyperoxia-Exposed Immature Rats*

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Asthma and bronchopulmonary dysplasia are prevalent diseases characterized by airway constrictor hyperresponsiveness and excessive airway smooth muscle accumulation. It is now well accepted that some alterations in airway wall architecture can increase luminal narrowing for a given degree of smooth muscle activation. For example, increasing the thickness of the submucosal layer between airway smooth muscle and airway lumen can magnify the airflow obstruction induced by a fixed proportion of airway smooth muscle shortening, an effect especially pronounced in smaller airways. Also, structural changes that result in thickening of the adventitial portion of an airway might lead to excessive luminal narrowing on smooth muscle activation, by reducing (or uncoupling) the external load ordinarily applied by the lung parenchyma to the airway through alveolar septal attachments that surround the circumference of intrapulmonary airways.

Despite this interest in structure-function relationships of airways remodelled by disease, rather less attention has been paid to the possibility that increased airway smooth muscle thickness could lead to increased force generation upon activation by constrictor stimuli, and that this increased force might lead to excessive luminal narrowing and airflow obstruction, even if other functional and structural features were normal. Indeed, computational models used initially to test the influence of submucosal thickening reveal that increasing smooth muscle force generation (as might occur with increased smooth muscle thickness) could be a most effective way of increasing airway constrictor responsiveness.

To test whether increased airway smooth muscle content could lead to airway constrictor hyperresponsiveness, we sought to identify an animal model which exhibited both these structural and functional abnormalities, in order to test whether the functional hyperresponsiveness depends causally upon the structural airway remodelling. Based upon the possible role of hyperoxia exposure in bronchopulmonary dysplasia, we tested the influence of hyperoxia exposure on immature rats.

In this model system, 21 day-old Sprague-Dawley rats were exposed to >95% oxygen for 8 days; control rats were similarly treated but were air-exposed. After exposure (on

FIGURE 1. Typical periphera airways from immature rats exposed to air (top) or oxygen (bottom) for 8 days. Note the substantial thickening of airway epithelial and smooth muscle layers in the hyperoxia-exposed animal (hematoxylin-eosin, original magnification X125; adapted from Hershenson et al9).

FIGURE 2. Peripheral airways from immature rats exposed to air (top) or oxygen (bottom) for 8 days. Left (top and bottom) show histologic sections (Gomori's trichrome plus aldehyde fuchsin, original magnifications X125. Right (top and bottom), corresponding contour tracings performed during computer-assisted morphometric analysis. E=epithelial layer; SM=smooth muscle layer (adapted from Hershenson et al9).
day 29 after birth), animals were studied physiologically and/or killed for histologic analysis. Oxygen-exposed immature rats developed substantial thickening of the airway wall (Fig 1) within the small peripheral and medium-sized central conducting airways. Connective tissue staining followed by morphometric analysis (Fig 2) showed that thickening occurred in both the epithelial and smooth muscle layers of the airway wall (Fig 3). Both light microscopy (Fig 1) and electron microscopy (Fig 4) suggested that increased numbers of cells were present in each layer.

To test whether increased cellular proliferation contributed to the airway wall thickening observed in hyperoxia-exposed immature rats, bromodeoxyuridine was administered (BrDu, a thymidine analog whose incorporation into DNA can be detected immunohistochemically) to experimental animals to label the nuclei of those airway cells that have undergone S-phase during the period of BrDu exposure. Time course studies demonstrated that histologic changes were first evident on day 6 of oxygen exposure (Fig 5); consequently, we administered BrDu for 48 h on days 3 to 4, 5 to 6, or 7 to 8 of oxygen or air exposure, and analyzed the fraction of small airway epithelial or smooth muscle layer cells that stained positively for BrDu. As shown in Figure 6, both airway epithelial and smooth muscle layer cells exhibited increased S-phase traversal after 6 or 8 days of oxygen exposure compared with air-exposed control animals. This finding implicates excess cell proliferation in the airway wall remodelling caused by hyperoxia-exposure.

One possible explanation for increased cellular proliferation might be that these airway cells are bathed in an environment containing excessive mitogenic activities for airway epithelial cells or airway smooth muscle cells. To test this hypothesis, we evaluated mitogenic activity within bronchoalveolar lavage fluid (BAL) obtained from immature rats after 8 days of oxygen or air exposure. Thus far, we have limited our evaluation to assessment of mitogens for airway mesenchymal cells. Preliminary results demonstrate that BAL from hyperoxic animals causes excess...
Figure 5. Time course of changes in mean small airway epithelial (top) and smooth muscle (bottom) layer thickness during exposure of immature rats to air (broken lines and open circles) or oxygen (solid lines and closed circles). Asterisk designates significant difference from day 0 value.

Figure 6. Fractional bromodeoxyuridine (BrDu) labelling of cells in the epithelium or smooth muscle layers of small airways from immature rats exposed to air (open circles) or oxygen (closed circles) for the number of days shown. Asterisk designates significant difference between air and oxygen exposure groups. Oxygen exposure increased epithelial and smooth muscle layer cellular S-phase traversal.

Figure 7. Tritiated thymidine incorporation into cultured BAL-Be/c3T3 cells exposed to BAL fluid from immature rats exposed for 8 days to air (open bars) or oxygen (filled bars), or to 1% or 10% fetal calf serum as shown. BAL fluid was diluted with serum-free culture medium as shown. Bronchoalveolar lavage fluid from hyperoxia-exposed immature rats exhibited mitogenic activity for 3T3 cells in a concentration-dependent fashion.

Figure 8. Dose response curves presenting severity of bronchoconstriction caused by inhalation of aerosolized methacholine at varying concentrations in air-exposed (open circles) or hyperoxia-exposed (closed circles) immature rats. Rrs experimental=respiratory system resistance following methacholine inhalation; Rrs baseline=respiratory system resistance prior to aerosol exposure; BL=baseline; SAL=saline inhalation (from Hershenson et al).
tritiated thymidine incorporation in cultured BALB/c/3T3 cells, a mesenchymal cell line used as a surrogate target in our initial experiments (Fig 7). The mitogenic activity for 3T3 cells is lipid-inextractable and is trypsin-digestable and concentrates with the >10 kd fraction when subjected to centrifugal filter concentration. So, it is likely that the mitogenic activity identified for 3T3 cells is a polypeptide growth factor. Characterization of this growth-promoting activity is now underway. Subsequent studies employing culture rat tracheal smooth muscle provide generally similar results, and preliminary evaluation of the nature of the growth factor suggests a possible role for thrombin or some other Gi-coupled serine protease.

But does the increased smooth muscle found in hyperoxia-exposed immature rats actually cause bronchoconstrictor hyperresponsiveness? Initial experiments documented clearcut airway constrictor hyperresponsiveness in hyperoxia-exposed immature rats (Fig 8). Subsequently, we exploited the variability of magnitude of airway remodelling among individual hyperoxia-exposed animals (Fig 3) to test whether the severity of airway hyperrespon-

**Figure 9.** Correlations between airway responsiveness, expressed as the log estimated concentration of aerosolized acetylcholine required to double respiratory system resistance (EC200 ACh), and mean thickness of small or central airway epithelial or smooth muscle layers, in five air-exposed (open circles) and 14 oxygen-exposed (closed circles) immature rats. Airway reactivity increased (reflected in lower log EC200 ACh values) with the severity of airway remodelling (from Hershenson et al).

**Figure 10.** Bronchoconstriction dose-response curves elicited by administration of increasing concentrations of aerosolized acetylcholine (Ach) in immature rats at various times following 8 days air (left) or oxygen (right) exposure. Circles=immediately following air or oxygen exposure; triangles=16 days after cessation of air or oxygen exposure; squares=48 days after air or oxygen exposure. There is no substantial change in airway reactivity after air exposure, but airway constrictor hyperresponsiveness present immediately after oxygen exposure resolves with time after cessation of oxygen exposure (from Hershenson et al).

**Figure 11.** Small airway epithelial and smooth muscle layer thicknesses in immature rats at various times following 8 days air (open bars) or oxygen (solid bars) exposure. There is no substantial change in airway layer thickness after air exposure, but airway layer thickening present immediately after oxygen exposure resolves with time after cessation of oxygen exposure (data are from Hershenson et al).

**Figure 12.** Active stress generation in vitro by tracheal rings obtained from air-exposed (open circles) or hyperoxia-exposed (closed circles) immature rats, in response to addition of increasing concentrations of acetylcholine (Ach) to the organ bath. Left, tracheal rings studied with epithelium intact; right, tracheal rings studied with epithelium removed. Tracheal rings from hyperoxia-exposed animals generated greater stress than did rings from air-exposed animals, and this effect was ablated by removal of the airway epithelium (adapted from Hershenson et al).
sponsiveness in vitro correlates with the severity of airway remodelling among individual animals. As shown in Figure 9, the severity of the functional abnormality (i.e., airway constrictor hyperresponsiveness), manifest as lowering of the ED200ACh (the estimated dose of acetylcholine needed to double respiratory constrictor in vivo) correlates with thermore, when the ED200ACh is reduced, the severity of hyperresponsiveness and their airway wall layer thickening resolve toward normal in parallel (Fig 10 and 11). This constant association between the magnitudes of hyperoxia-induced structural and functional airway abnormalities during both peak and resolution phases supports, though does not prove, the notion that hyperoxia-induced airway remodelling causes airway constrictor hyperresponsiveness in immature rats. A more definitive test of this potential causal relationship may come when, after identifying the precise nature of the mitogenic activity in hyperoxic animals’ BAL fluid, specific interventions are designed to neutralize this activity during oxygen exposure. Still, it seems unlikely that airway remodelling can entirely explain hyperoxia-induced airway constrictor hyperresponsiveness, as we have also demonstrated epithelium-dependent increases in tracheal smooth muscle stress generation in vitro, in tissues obtained from hyperoxia-exposed rats (Fig 12). Thus, hyperoxia-induced airway constrictor hyperresponsiveness appears to be the complex result of multiple simultaneous mechanisms, which may include airway remodelling as a principal contributor.

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Chronic Allergic Inflammation Induces Replication of Airway Smooth Muscle Cells In Vivo in Guinea Pigs*

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The hypothesis that chronic antigen challenge induces airway smooth muscle proliferation in guinea pig airways was tested. We quantified proliferating airway smooth muscle cells incorporating the thymidine analogue 5′-bromo-2′-deoxyuridine (BrDu). The BrDu, 25 mg/kg, was administered intraperitoneally 2 times a week for 5 weeks and daily in the sixth week to 10 control (CON) adolescent guinea pigs and to 16 guinea pigs sensitized to ovalbumin (OA). The OA animals were exposed twice weekly to nebulized 0.5% OA and control animals were given nebulized saline solution. Lungs were processed for histologic findings after physiologic measurements had been obtained.

Maximal pulmonary resistance increased twofold and the acetylcholine (ACh) concentration causing a tenfold increase in pulmonary resistance decreased fourfold in the OA group as compared to the CON group. In vitro maximal smooth muscle stress values in response to 1mM ACh increased twofold in the OA group as compared to the CON group, but the smooth muscle mass was not increased. Serial sections of membranous airways were stained with hematoxylin-eosin or for BrDu by immunohistochemistry. The airway smooth muscle area and nuclei were point-counted, and the smooth muscle cell proliferation index was calculated as the percent of BrDu-labelled nuclei/total nuclei. The smooth muscle proliferation index was increased (OA=24.4 ± 6.2% (SEM) vs CON=3.9 ± 1.5%, p<0.02). An increase in BrDu-positive granulocytes (predominantly eosinophils) was noted in the mucosa and adventitia but not in the smooth muscle.

These results are consistent with the notion that airway smooth muscle proliferates as part of the chronic allergic inflammatory response. Increased smooth muscle DNA replication without a measurable increase in muscle area suggests either an early hyperplastic response or increased cell turnover.

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