Biochemical and Morphologic Responses of Rat Nasal Epithelia to Hyperoxia

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Biochemical and Morphologic Responses of Rat Nasal Epithelia to Hyperoxia. NIKULA, K. J., SABOURIN, P. J., FRIETAG, B. C., BIRDWHISTELL, A. J., HOTCHKISS, J. A., AND HARKEMA, J. R. (1991). Fundam. Appl. Toxicol. 17, 675-683. While performing its functions in olfaction, modification of inspired air, and protection of the lower respiratory tract from high concentrations of potentially harmful inhalants, the nasal mucosa can be injured by a number of inhalants. In this study, F344/N male rats were exposed to filtered air or hyperoxia (85 or 87% oxygen), 24 hr/day, 7 days/week, for 1 (acute exposure) or 11 (chronic exposure) weeks. There were distinct differences between the different epithelial regions examined in replicative and morphologic responses as well as altered enzyme activities in response to oxygen exposure. Neither acute nor chronic hyperoxic exposure caused degenerative, necrotizing, or inflammatory changes in any of the nasal epithelium examined. Hyperoxia-induced hypertrophy, but not hyperplasia, of the non-ciliated cuboidal (NCC) epithelium occurred after both acute and chronic exposure. Cell replication was increased in portions of the NCC and respiratory epithelia after acute hyperoxia exposure. There were significant increases, compared to controls, in the specific activity of glucose-6-phosphate dehydrogenase in the nasal turbinates, maxilloturbinates, and lateral wall epithelium (NCC epithelium), the nasal septum (respiratory epithelium), and the ethmoturbinates (olfactory epithelium), and in the specific activity of glutathione peroxidase in the NCC epithelium and ethmoturbinates after acute hyperoxia exposure. The specific activity of cytochrome P450-dependent monooxygenase-catalyzed O-deethylation of 3-cyano-7-ethoxycoumarin was significantly decreased, compared to controls, in the NCC epithelium. These results suggest that hyperoxia exposure induces morphologic and biochemical alterations in nasal epithelia which appear to be protective responses of certain cell types to hyperoxia.

The functions of the nasal cavity and mucosa are (1) olfaction, (2) warming and humidification of inspired air, and (3) protection of the lower respiratory tract from high concentrations of potentially harmful inhalants. While serving these roles, the nasal mucosa can be injured by a number of inhalants (Harkema, 1990). One potentially toxic inhalant is oxygen at high concentration. The nasal epithelia of people receiving oxygen therapy may be exposed to up to 100% oxygen by intranasal cannulas. These exposures are often for only a few days, but some patients receive oxygen therapy for several months. The human nasal cavity may also be exposed to an enriched oxygen atmosphere in space or during deep sea exploration (Frank, 1985).

The biochemical and morphologic effects of high concentrations of normobaric (<760 mm Hg) oxygen on the lung have been well documented in both humans and laboratory animals (reviewed in Frank, 1985; Clark and Lambertsen, 1971; Fisher et al., 1984; Fisher...
and Forman, 1985). The acute morphologic effects of pulmonary oxygen toxicity are primarily injury to endothelial and type I cells and edema. Subacute-to-chronic changes include repair of the damaged endothelium, hypertrophy and hyperplasia of type II cells, proliferation of interstitial cells, and deposition of collagen and elastin (Frank, 1985; Clark and Lamberts, 1971). Acute-to-subacute exposure of rats to sublethal hyperoxia (for example, to 80% O2 for 7 to 14 days) causes increased activity of pulmonary superoxide dismutase, catalase, glutathione peroxidase, glutathione reductase, and glucose-6-phosphate dehydrogenase, as well as an increase in the nonprotein sulfhydryl content of the lung (Coursin et al., 1987; Freeman et al., 1986).

The nasal and pulmonary morphologic effects of another oxidant gas, ozone, have also been extensively studied (reviewed in Har-kema, 1990; Menzel, 1984). Acute and chronic ozone exposure causes hyperplasia and secretory metaplasia in the nonciliated cuboidal epithelium and foci of deciliation in the respiratory epithelium within the proximal aspect of the nose of both monkeys and rats. Acute ozone exposure damages type I and ciliated cells and induces proliferation of type II cells within the lungs of both laboratory rodents and nonhuman primates. Chronic ozone exposure inhibits ciliagenesis and type II cell maturation. The location and intensity of the damage depends on the ozone concentration. The biochemical effects of ozone exposure have been studied in the lung (Jackson and Frank, 1984; reviewed in Menzel, 1984), but not in the nose. As with hyperoxia, the activities of the enzymes of the antioxidant protective system, including glutathione peroxidase, glutathione reductase, superoxide dismutase, catalase, and glucose-6-phosphate dehydrogenase, are increased in lungs exposed to ozone, as is the content of nonprotein sulfhydryl.

The morphologic effect of hyperoxia on the nasal epithelia has not been previously examined, nor have antioxidant enzyme activities been measured in nasal tissue after hyperoxia exposure. The nasal cavity is lined by four types of epithelia: (1) squamous, (2) nonciliated cuboidal, (3) respiratory, and (4) olfactory. The purpose of this study was to examine (1) the effects of acute and chronic hyperoxia on nasal epithelial morphology and (2) the effects of hyperoxia on antioxidant enzyme and cytochrome P450-dependent mono-oxygenase activities in nasal tissue.

**MATERIALS AND METHODS**

*Animals* Male F344/N rats from the Inhalation Toxicology Research Institute’s colony were used in these studies. The rats were conditioned in whole-body exposure chambers supplied with filtered air for 2 weeks prior to assignment to treatment groups. For the initial, 11-week-exposure studies, eleven 10- to 11-week-old rats were randomized by body weight and assigned to one of two treatment groups: (1) five rats were exposed to filtered ambient air (11-week controls) and (2) six rats were exposed to hyperoxia (11-week hyperoxia). At the time of termination, rats from each group were again randomized by body weight and subdivided into sets; 40 were used for the enzymology portion of the study, 6 for the light microscopy and morphometry studies, and 3 for the ultrastructural studies.

*Exposures and animal maintenance.* The rats were housed individually in rack-mounted, stainless steel wire cages within whole-body chambers (HC-1000, Hazleton Systems, Lenexa, KS). The 11-week exposures were to filtered, ambient air (1-week controls) and 49 were exposed to hyperoxia (11-week hyperoxia). At the time of termination, rats from each group were again randomized by body weight and subdivided into sets; 40 were used for the enzymology portion of the study, 6 for the light microscopy and morphometry studies, and 3 for the ultrastructural studies.

*Exposures and animal maintenance.* The rats were housed individually in rack-mounted, stainless steel wire cages within whole-body chambers (HC-1000, Hazleton Systems, Lenexa, KS). The 11-week exposures were to filtered, ambient air or 85 ± 3% oxygen (average barometric pressure 620 Torr, Albuquerque, NM). All exposures were for 24 hr/day, 7 days/week, except during the time required for twice-daily cage cleaning and animal maintenance. The oxygen was supplied from tanks of liquid oxygen diluted to 85 or 87% with filtered air. The flow rate through the chambers was 134 liters/min (eight chamber volume changes/hr). Oxygen concentration in the chambers was continuously monitored with a portable, alarm-capable, oxygen monitor and recorded three or four times daily.

The rats were supplied with food (Wayne Certified Lab-Blox, Allied Mills, Chicago, IL) and water ad libitum, and were maintained on a 12-hr light/12-hr dark cycle, starting at 6 AM, at a temperature of 21–25°C and a relative humidity of 40–70%.

*Morphology and morphometry—11-week studies.* The rats used for these studies were terminated by injecting sodium pentobarbital, 250 mg/kg, intraperitoneally. The
head of each rat was removed from the carcass and the airways were flushed retrograde through the nasopharyngeal orifice with 10 ml of 10% neutral buffered formalin. The eyes, lower jaw, skin, and musculature were removed, and the head was immersed in approximately 250 ml of 10% neutral buffered formalin for 4 days, after which it was decalcified in a 13% formic acid solution for 3 days, rinsed in tap water for 4 hr, and sectioned transversely to produce four tissue slices (Young, 1981) (T1–T4, Fig. 1A). These tissue slices were dehydrated in a graded series of ethanol and embedded in paraffin. A 5-μm-thick section was cut from the anterior surface of each tissue block and was stained with hematoxylin and eosin. These sections were examined by light microscopy. Alterations were only observed in the section from the first tissue block. In order to obtain a thinner section for morphometric analysis, this tissue block, designated T1, was deparaffinized and was embedded in Poly-Rec (Polysciences, Inc., Warrington, PA) embedding medium. A 1-μm-thick section was cut from the anterior face of T1, the embedding resin was extracted with acetone, and the section was rehydrated and stained with toluidine blue (Fig. 1B).

The T1 sections were examined qualitatively, and the nonciliated cuboidal (NCC) epithelium of the lateral aspect of the nasal turbinate, the lateral aspect of the maxilloturbinate, and the lateral wall were examined morphometrically. We used a semiautomatic image analysis system to determine both the number of epithelial nuclei/mm of basal lamina and the arithmetic mean thickness of the epithelium (Elias and Hyde, 1983) in one section from each animal. The slides were imaged with a 40× planapo objective and a 1.25× intermediate lens (Olympus Optical Co., Tokyo, Japan) with a CCD camera (TM-840, Pulnix America, Sunnydale, CA) and a FG-100 digital image processing board (Imaging Technology, Inc., Woburn, MA) and were displayed on a color monitor. The tissue sections were examined at a final magnification, on the monitor, of 2500×.

Morphology, morphometry, and immunohistochemistry—1-week studies. Two hours prior to sacrifice (8:00 AM–10:00 AM), the rats used in these studies were injected intraperitoneally with 50 mg/kg body weight of 5-bromo-2′-deoxyuridine (BrdU, Sigma, St. Louis, MO), a thymidine analog (Johnson et al., 1990). All rats were euthanized between 10:00 AM and noon, anesthetizing with 5% halothane in O₂ and then exsanguinating via the abdominal aorta. The nasal cavities were fixed as described for the 11-week studies, except that Carnoy's fixative (Johnson et al., 1990) was used. Following decalcification in 13% formic acid, the T1 block was processed and embedded in Poly-Rec. The anterior surface was sectioned at 1 μm and the sections were processed and stained as described for the 11-week studies. The tissue block anterior to T1, designated T0 (Fig. 1A), was embedded in paraffin. Five-micrometer sections were cut from the caudal face of T0 (Fig. 1B), deparaffinized, and a previously described immunohistochemical method was used to detect BrdU incorporated into nuclei (Johnson et al., 1990).

The epithelia examined were (1) the NCC epithelium of the lateral aspect of the nasal turbinate, lateral wall, and the lateral aspect of the maxilloturbinate; (2) the respiratory epithelium of the nasal septum; and (3) the olfactory epithelium of the dorsal meatus. The number of epithelial nuclei/mm of basal lamina and the arithmetic mean thickness of each of the three epithelia were determined in one T1 section from each rat. We used the T0 section from each animal to determine the number of BrdU-labeled (S-phase) nuclei/mm of basal lamina (unit length labeling index) (Johnson et al., 1990; Monticello et al., 1990) for each of the three epithelia. The semiautomated image analysis system was used for the morphometric analyses, as described earlier, except that a light microscope (100X oil immersion objective lens, 10X ocular lens, Olympus BH-2, Olympus Optical Co) was used to count the BrdU-labeled nuclei. For the NCC epithelium, we re-
corded the results for each of the three subregions (nasal turbinate, maxilloturbinate, lateral wall) separately.

Ultrastructural examination—1-week studies. The rats used for these studies were sacrificed by means of 5% halothane in oxygen anesthesia and exsanguination. The nasal cavities were fixed as described previously, but with Karnovsky's fixative. After fixation, the NCC epithelium of the nasoturbinate was removed and embedded in Epon-Araldite (Ted Pella, Inc., Redding, CA). Thin sections of the epithelium were placed on copper hexagonal mesh grids (Polysciences), poststained with uranyl acetate and Reynold's lead citrate, and examined with a Hitachi 7000 STEM electron microscope.

Enzymatic assays. The rats used for the enzymatic assays were anesthetized with carbon dioxide and decapitated. The head was split open sagitally. Samples of nasal tissues from three locations, (1) the nasal septum, (2) the nasoturbinate, maxilloturbinate, and lateral wall epithelium, and (3) the ethmoturbinates (Fig. 2), were immediately removed from each rat and placed in 10 mM potassium phosphate buffer, pH 7.5, at 4°C. Within each exposure group, four pooled samples of each of the three tissue regions were formed by grouping the like tissues from ten rats into a single sample. The four samples were weighed, and 8 vol of buffer were added (ml/g). The samples were then homogenized with a Polytron homogenizer and centrifuged at 1000g for 10 min. One-fifth of each supernate was used for the superoxide dismutase assay. The remainder of each supernate was centrifuged at 9000g for 10 min. Each supernate was then divided into aliquots for the remaining assays. All aliquots not used immediately for assays were bubbled with nitrogen gas, frozen in liquid nitrogen, and stored at -70°C for future use. Aliquots were freeze-thawed only once prior to being assayed.

Previously published methods were used to determine specific enzyme activities and protein concentrations. Superoxide dismutase was measured by the inhibition of the autoxidation of epinephrine to adrenochrome at pH 10.2, a reaction mediated by the superoxide radical (Misra and Fridovich, 1972). Catalase was measured as described by Tolbert (1974), except that the Triton X-100 was used at 0.1% instead of 0.01%. Glutathione peroxidase was measured by the method of Paglia and Valentine (1967), as modified by Cohen and Rosemeyer (1975), but in a total assay volume of 1 ml, instead of 3 ml. Cytochrome P450 activity was measured by the O-deethylation of 3-cyano-7-ethoxycoumarin (White, 1988). Protein concentration was determined by the Lowry method (Lowry et al., 1951), as modified by Bieber et al. (1972).

The activity level for each enzyme was determined in each tissue sample. The results for each sample were normalized by dividing the activity by the protein concentration. The mean specific activity and standard deviation for each tissue type were calculated by using the specific activities for the four samples isolated from each tissue.

![Figure 2: Sagittal sections of the rat nasal cavity. The nasal septum (stippled) is in place in (A). The darkly stippled area corresponds to the portion sampled for enzymology. The nasal septum has been removed in (B) to reveal the underlying nasal (N) and maxillo- (M) and ethmo- (E) turbinates. The stippled areas correspond to the portions sampled for enzymology. The NCC epithelium lateral to the stippled portions of the naso- and maxilloturbinates was stripped from the lateral wall and included in the NCC sample.](https://academic.oup.com/toxsci/article-abstract/17/4/675/1638806)

Statistical analysis. The data were tested for equality of group means with an unpaired Student's t test. The criterion for statistical significance was set at \( p < 0.05 \).

RESULTS

Morphology, Morphometry, and Immunohistochemistry

After 11 weeks of hyperoxic exposure, the cells of the NCC epithelium had changed from cuboidal to columnar (Fig. 3). There were no apparent morphologic differences between the control and the hyperoxia-exposed respiratory, olfactory, and squamous epithelia. Oxygen exposure did not induce degenerative, necrotizing, or inflammatory changes in any of the epithelia examined.

Because the NCC epithelium was the one epithelial type exhibiting a morphologic alteration, this epithelium was examined morphometrically. There were no significant differences between control and exposed NCC epithelia in the number of epithelial nuclei/mm
basal lamina. There was a significant (65–100%) hyperoxia-induced increase in the arithmetic mean thickness of the NCC epithelium (Fig. 4).

One week of hyperoxic exposure did not induce degenerative, necrotizing, or inflammatory changes observable by light microscopy nor were there significant alterations in the number of epithelial nuclei/mm basal lamina in any of the epithelia examined. There was a significant (25 to 50%) hyperoxia-induced increase in the arithmetic mean thickness of the NCC epithelium (Fig. 4), but not of the respiratory or olfactory epithelia (Fig. 5).

After 1 week of hyperoxic exposure, there were more BrdU-labeled nuclei/mm basal lamina in the epithelia of the nasal turbinate (NCC), maxilloturbinate (NCC), and nasal septum (respiratory) than in the same epithelia

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**Fig. 3.** Representative light micrographs (×800) of the nonciliated cuboidal epithelium from control (A) and hyperoxia-exposed (B) rats.

**Fig. 4.** Histogram showing the arithmetic mean thickness of the nonciliated cuboidal epithelium. There were no significant differences between the 1- and 11-week controls in the mean thickness of each region, so the control values were pooled. Significant increases were seen in all three subregions after 1 and 11 weeks of exposure. *Significantly different from control, p ≤ 0.05. †Significantly different from 1-week exposed, p ≤ 0.05. Data represent means ± SD.

**Fig. 5.** Histogram showing the arithmetic mean thickness of the respiratory and olfactory epithelia. The mean thickness was not increased after 1 week of hyperoxic exposure. Data represent means ± SD.
from control animals. There was no increase in the unit length labeling index of the epithelium of the dorsal meatus (olfactory) or the lateral wall (NCC) (Fig. 6).

Ultrastructurally, the cytoplasm of the hyperoxia-exposed, hypertrophic, epithelial cells of the NCC contained numerous, elongate mitochondria (Fig. 7).

**Enzymology**

After 1 week of hyperoxic exposure, there were significant increases, compared to controls, in the specific activity of glucose-6-phosphate dehydrogenase in the nasal turbinates, maxilloturbinates, and lateral wall epithelium (NCC epithelium), the nasal septum (respiratory epithelium), and the ethmoturbinates (olfactory epithelium), and in the specific activity of glutathione peroxidase in the NCC epithelium and ethmoturbinates. The specific activity of cytochrome P450-dependent monooxygenase was significantly decreased, compared to controls, in the NCC epithelium (Fig. 8).

**DISCUSSION**

The number of epithelial nuclei/length of the basal lamina reflects the number of cells/surface area of the basal lamina. The arithmetic mean thickness of the epithelium is equivalent to the volume of the epithelium/surface area of the basal lamina. Because the number of cells/surface area of the basal lamina did not change with hyperoxic exposure, but the volume of the epithelium/surface area of the basal lamina increased in the NCC epithelium, the volume of the cells must have increased. Thus, the hyperoxic exposure induced hypertrophy, but not hyperplasia, of the NCC epithelium. The other possible explanation for the volume change is that the hyperoxic exposure caused cell swelling. However, the ultrastructural evaluation showed an increase in cytoplasmic organelles, particularly mitochondria, and no increase in electron-lucent, organelle-free cytoplasm, nor any evidence of swelling of organelles. Thus, we concluded that the volume change was due to cell hypertrophy and not cell swelling.

The increase in S-phase nuclei suggests that cell turnover was increased in three of the five regions or subregions examined after 1 week of exposure. Because there was no hyperplasia after 1 or 11 weeks of exposure, cells damaged by hyperoxia were most likely replaced by replication, without increasing the total number of cells in that compartment; i.e., there was no proliferation.

There are both similarities and differences between the responses of the rat NCC and respiratory epithelium to either hyperoxia or 0.8 ppm ozone exposure (6 hr/day) for 7 days. Both types of oxidant exposures cause an increase in cell replication in the NCC, as measured by BrdU incorporation. However, the following differences are observed: (1) hyperoxia also causes an increase in replication in the respiratory epithelium, (2) ozone exposure causes a hyperplastic response in the NCC (Johnson et al., 1990), and (3) ozone exposure induces secretory metaplasia of the NCC epithelium of the maxilloturbinates (Harkema et al., 1989). These similarities and differences in the responses to two different oxidants show...
that, while the NCC may have a limited variety of possible responses to injury, these responses do vary according to the specific toxicant involved.

There is an increase in oxidant-protective enzyme activity in the nasal tissues after exposure to hyperoxia. This increased activity could be due to either increased enzyme production or to increased enzyme reactivity.

It appears that, with hyperoxic exposure, induction of the glutathione peroxidase pathway is important in protecting against reactive oxidant species. Both glutathione peroxidase and glucose-6-phosphate dehydrogenase were significantly increased, but superoxide dismutase and catalase were not. This observation could be further verified by assaying for increased glutathione reductase activity, because its activity is coupled to that of glutathione peroxidase and glucose-6-phosphate dehydrogenase.

Hypertrophy of the NCC epithelium would have been accompanied by an increase in protein content. Thus, the magnitude of the increase in enzyme activity may have been diminished by normalizing to the protein concentration. If the enzyme activity had been normalized to DNA content, as an expression of activity/cell, then the increased activities of glutathione-6-phosphate dehydrogenase and glutathione peroxidase in the NCC epithelium would have been even larger, compared to controls. Likewise, the decrease in the specific activity of the cytochrome P450-dependent monooxygenase in the NCC epithelium probably reflects the increase in nonmicrosomal protein. If this enzyme activity had been normalized to DNA content, it probably would not have been altered, or may have increased, compared to controls.

Previous reports have shown that the normal rat anterior nose contains four distinct,
morphological epithelial regions: squamous; ciliated respiratory; nonciliated cuboidal; and olfactory (Johnson et al., 1990), and that these regions respond differently to inhaled toxicants such as ozone (Johnson et al., 1990), formaldehyde (Chang et al., 1989), dichloropropene (Stott et al., 1988), and tobacco smoke (Johnson et al., 1989). Also, the distinct differences in replicative and morphologic responses as well as altered enzyme activities, in response to oxygen exposure, further emphasize the differences between these epithelia.

In summary, hyperoxia induces hypertrophy, but not hyperplasia, of the NCC epithelium in rats. Ultrastructurally, there was no evidence of cell swelling. The cytoplasm contained numerous mitochondria, many of which were elongate. Pleomorphic, elongated mitochondria were seen in type II cells of oxygen-tolerant rats (85% O$_2$, 7 days), and it was suggested that the mitochondrial change could protect against oxygen toxicity by providing increased surface/volume ratios that compensated for the direct inhibition of enzymatic activity (Rosenbaum et al., 1969). Antioxidant enzyme activities in rat nasal epithelia were increased by hyperoxic exposure. The increase in cell replication seen in the respiratory and NCC epithelia after 1 wk of hyperoxic exposure may reflect injury to sensitive epithelial cells and replacement of these cells by more resistant cells. In the olfactory epithelia, the increase in antioxidant enzyme activity was not accompanied by a detectable increase in replication 1 week after exposure, nor was there hyperplasia or hypertrophy.

These results suggest that oxygen therapy may induce morphologic and biochemical alterations in the nasal epithelia, which appear to be the protective responses of certain cell types to hyperoxia. The effects of these adaptations on air flow, clearance, xenobiotic metabolism, and response to other oxidants remain to be determined.

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