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# Effect of Ozone Treatment on Airway Reactivity and Epithelium-Derived Relaxing Factor in Guinea Pigs[1]

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## ABSTRACT

Ozone ($O_3$) is toxic to respiratory epithelium and causes airway inflammation and hyperreactivity. To evaluate the role of the epithelium in the development of hyperreactivity, we examined in guinea pigs the effects of inhaled $O_3$ (3 ppm for 1 h; 0–24 h after exposure) on 1) reactivity to inhaled methacholine (MCh), 2) reactivity of the isolated, perfused trachea (IPT) to MCh, 3) epithelium-derived relaxing factor (EpDRF)-mediated relaxations of IPT induced by mucosal hyperosmolar solutions, 4) neurogenic contraction and relaxation responses, 5) transepithelial potential difference, and 6) microscopic analysis of nitrotyrosine immunofluorescence, substance P fiber density, and tracheal morphology. At 0 h, $O_3$ caused hyperreactivity to inhaled MCh and mucosally but not serosally applied MCh in IPT (only in the presence of the epithelium) and a decrease in transepithelial potential difference. Inhibition of EpDRF-induced relaxation responses occurred at 2 h. All of these changes returned to control by 12 to 18 h. $O_3$ had no effect on neurogenic responses. Nitrotyrosine immunofluorescence appeared in the trachea at 0 h in detached epithelial cell ghosts and in intrapulmonary airways by 6 h. Substance P fiber density was elevated in smooth muscle at 0 and 18 h but not in epithelium or lamina propria of intrapulmonary and extrapulmonary bronchi. Loss of cilia and mucosubstances in the mucosa occurred at 0 h; the epithelium became markedly attenuated over 12 to 24 h. A reversible increase in epithelial permeability and a decrease in EpDRF production may contribute to $O_3$-induced hyperreactivity to MCh.

Inhalation exposure to ozone ($O_3$) causes several adverse effects in the lung. Typically, the forced expiratory volume in 1 s is reduced, airway reactivity to bronchoconstrictors such as methacholine (MCh) is increased, and neutrophilic inflammation occurs in the airways (American Thoracic Society, 1996). Responses of the airways to $O_3$ occur in three phases: immediate (0–2 h), early (2–24 h), and late (12–24 h) (Leikauf et al., 1995; American Thoracic Society, 1996). An accumulation of polymorphonuclear leukocytes (PMNs) in the mucosa and submucosa and transit of the cells into the air space occur during the early phase (Schultheis and Bassett, 1994; Pendino et al., 1995) and may be transient after acute exposure.

$O_3$ causes morphological damage to respiratory epithelium; this effect occurs before the inflammatory response, and it could contribute to the development of airway hyperreactivity (Murlas and Roum, 1985). Murlas et al. (1990) observed that hyperresponsiveness of tracheal rings in vitro to contractile agonists occurred only in the presence of the epithelium. $O_3$ causes an increase in epithelial permeability, which, depending on the exposure protocol, may recover within 1 day (Kleeberger and Hudak, 1992; Young and Bhalla, 1992). Guinea pigs became hyperreactive to inhaled but not intravenously administered histamine or MCh (Yeadon et al., 1992; Matsubara et al., 1995), suggesting that the epithelial diffusion barrier to air-borne agents had been compromised. Inhibition of neutral endopeptidase by $O_3$ may contribute to hyperreactivity to neuropeptides such as substance P, as well as to histamine and cholinergic agonists (Murlas et al., 1990; Yeadon et al., 1992; Loenders et al., 1996).

The airway epithelium has multiple roles in addition to serving as a diffusion barrier (Farmer and Hay, 1991a). For example, it regulates airway surface liquid and is a source of inflammatory mediators and prostanoids. In addition, the epithelium modulates the reactivity of airway smooth muscle

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[1] Mention of a brand name does not constitute product endorsement.

**ABBREVIATIONS:** MCh, methacholine; EpDRF, epithelium-derived relaxing factor; MKH, modified Krebs-Henseleit; PMN, polymorphonuclear leukocyte; IL, intraluminal; EL, extraluminal; $V_T$, transepithelial potential difference; EFS, electric field stimulation.

to contractile agonists and endogenous transmitters via the release of epithelium-derived relaxing factor (EpDRF), a nonprostanoid substance that is not nitric oxide (Munakata et al., 1990; Fedan et al., 1999) that reduces smooth muscle reactivity by hyperpolarizing the smooth muscle membrane (Flavahan et al., 1985; Fedan et al., 1988; Farmer and Hay, 1991b; Xie et al., 1992; Goldie and Hay, 1997) and is released by hyperosmolar solutions after bioelectric changes in the epithelium (Munakata et al., 1988; Dortch-Carnes et al., 1999; Fedan et al., 1999). Modulation of reactivity by the epithelium is itself regulated by unknown mechanisms, and compensatory increases and decreases in the inhibitory effect of epithelium on reactivity has been observed in animal models of pulmonary disease (Smith et al., 1993; Warner et al., 1996; Huang et al., 1997).

Here we used the toxicity of $O_3$ on respiratory epithelium as a treatment to alter airway reactivity in vivo and in vitro and examined concomitant changes in the modulatory effect of epithelium on reactivity. We compared these findings with changes in other systems in the airway that could influence reactivity, such as substance P and nitrotyrosine levels and efferent nerve activity. To examine alterations in epithelial function and EpDRF effects, we used the guinea pig isolated, perfused trachea preparation (Munakata et al., 1989; Fedan and Frazer, 1992). In this preparation, contractile agents such as MCh are more potent and efficacious when applied to the serosal surface, where they have free access to the smooth muscle, than they are after application to the mucosal surface, because of the epithelial diffusion barrier and the effects of the inhibitory substances originating in the epithelium (Munakata et al., 1989; Fedan et al., 1990, 1999; Fedan and Frazer, 1992). Relative mucosal versus serosal reactivity provides an index of the modulatory effect of the epithelium. The results suggest that $O_3$ leads to a decreased production and/or effect of EpDRF.

## Materials and Methods

**Animals.** All studies were conducted in facilities fully accredited by the Association for the Assessment and Accreditation of Laboratory Animal Care International (AAALAC). Male English short-hair SPF guinea pigs (400–600 g) were obtained from Harlan Sprague-Dawley, Inc. (Indianapolis, IN) and received feed and water ad libitum. The animals were anesthetized with sodium pentobarbital (65 mg/kg i.p.) and sacrificed under anesthesia by thoracotomy and bleeding.

**$O_3$ Exposure.** Conscious, unrestrained guinea pigs were placed in a stainless steel and glass chamber of ~27 liters and exposed to 3 ppm $O_3$ (Enmet Corporation, Ann Arbor, MI) in filtered, silica gel-dried air or filtered air (control animals) for 1 h. The $O_3$ level in the chamber was monitored (Columbia Scientific, Carrollton, TX) continuously and kept constant (±5%) by regulating dilutant air flow. This was done manually initially and later with the aid of a computer-controlled stepper motor connected to a flow regulator. Chamber air flow was ~ 3.8 l/min. Animals were examined immediately (0 h) or 2, 6, 12, 18, or 24 h after the conclusion of the exposure.

**Pulmonary Function and Reactivity to Inhaled MCh Aerosol.** Whole body plethysmography was used to examine the effects of $O_3$ treatment on pulmonary function and airway reactivity of conscious, unrestrained animals to MCh using measurement of enhanced pause (Penh; Buxco Electronics, Inc., Troy, NY) as an index of airway obstruction and effort of breathing, which is proportional to airway resistance (Chong et al., 1999). We observed previously (Lawrence et al., 1997; Rengasamy et al., 1999) that this method (Chand et al., 1993; Hamelmann et al., 1997; Chong et al., 1999) provides a reliable index of obstruction and airway reactivity in animal models of lung disease. Penh was derived from the relation $Penh = (T_e/T_r - 1)(PEF/PIF)$, where $T_e$ is expiratory time, $T_r$ is relaxation time (the time during expiration to reach a percentage of tidal volume which has not been expired), PEF is peak expiratory flow, and PIF is peak inspiratory flow. As discussed by Drazen et al. (1999), we observed changes in box pressure comparable with those depicted by Chong et al. (1999). Penh values were averaged over 10-s intervals and logged. After each animal experienced an acclimation period in air, Penh was recorded for at least 15 min to establish basal values. The animals were exposed to aerosolized (Ultra Neb; DeVilbiss, Somerset, PA) saline vehicle for 1 min, after which Penh was recorded for 15 min. To assess airway reactivity, MCh (Sigma Chemical Co., St. Louis, MO) was delivered via aerosol to generate dose-response curves. Each MCh aerosol dose was delivered for 1 min to the chamber, and the next higher concentration of MCh aerosol was delivered after at least 20 min or when Penh returned to the prechallenge level. The MCh solutions (in sterile saline) ranged from 0.01 to 1 mg/ml.

Basal pulmonary parameters and reactivity to MCh were measured on each animal 24 h before air or $O_3$ treatment and again at one time point per animal after treatment with air or $O_3$; that is, each animal served as its own control.

**Isolated, Perfused Trachea Preparation.** The isolated, perfused trachea preparation was used to examine airway reactivity to MCh applied to the mucosal surface [intraluminal (IL) bath] or to the serosal surface [extraluminal (EL) bath]. The preparation was also used to elicit EpDRF-mediated relaxation responses that are elicited by the IL application of hyperosmolar solutions (Munakata et al., 1988; Dortch-Carnes et al., 1999; Fedan et al., 1999).

As described previously (Munakata et al., 1988; Fedan and Frazer, 1992), a 4-cm segment of trachea was removed, cleaned, and mounted onto a perfusion holder that contained indwelling side-hole catheters that were connected to the positive (inlet) and negative (outlet) sides of a differential pressure transducer. The holder was placed into the EL bath containing modified Krebs-Henseleit (MKH) solution (37°C), and it was perfused (34 ml/min) with recirculating MKH solution (37°C) from a separate, 30-ml IL bath. Transmural pressure was adjusted to zero. Responses were measured as changes in the inlet minus outlet pressure difference (ΔP) in centimeters of $H_2O$. A 1-h equilibration period was allowed before the experiment, during which the MKH solution in both baths was changed at 15-min intervals.

**Measurement of Transepithelial Potential Difference ($V_T$).** To measure $V_T$ [equal to the sum of an apical and a basolateral potential ($V_a + V_b = V_T$) and an offset potential], tracheae were mounted onto a plastic perfusion holder similar to the one used for the measurement of ΔP. As described in detail previously (Dortch-Carnes et al., 1999), $V_T$ was recorded at the proximal end (inlet) of the trachea by placing voltage electrodes at the basolateral ($V_b$; EL bath) and apical ($V_a$; IL bath) surfaces of the trachea. $V_T$ was measured immediately after and 2.5 h after setting up the preparation in the bath. The EL and IL MKH solution was changed every 15 min during this period.

**Tracheal Strip Preparation.** Strips of trachea that were two cartilage rings wide were prepared as described previously (Fedan et al., 1986) and placed in organ chambers under 1 g resting force for the measurement of isometric contraction and relaxation responses. The preparations were equilibrated for 1 h before the experiment.

**Epithelium Removal.** To remove the epithelium from the trachea (Fedan and Frazer, 1992), before it was mounted to the perfusion apparatus, a 5- to 6-cm piece of trimmed pipe cleaner brush was advanced slowly into the lumen and withdrawn while rotating slowly.

**MCh Concentration-Response Curves.** Two cumulative MCh concentration-response curves were obtained from each perfused trachea preparation: first after additions to the EL bath and then

1.5 h later (washes every 15 min) after IL additions. None of the effects on MCh concentration-response curves shown in *Results* are observed in two, consecutively obtained control curves (Fedan and Frazer, 1992).

**Hyperosmolar NaCl Solution-Induced, EpDRF-Mediated Relaxation Responses.** As described previously (Fedan et al., 1999), concentration-response curves for relaxation responses to cumulatively added IL NaCl were generated after having obtained a stable contraction with EL MCh ($3 \times 10^{-7}$ M; $\sim EC_{50}$ value). The relaxations in response to NaCl were normalized as a percentage of the MCh-induced contraction. The NaCl concentrations shown in the abscissa of the figures refer to the molar concentrations added to the MKH solution. The osmolarity of MKH solution is 281.2 ± 0.6 mOsM ($n = 5$; Osmette A Automatic Osmometer; Precision Systems, Inc., Sudbury, MA), and $[Na^+]_{total}$ is 138 mM.

**Neurogenic Responses Elicited with Electric Field Stimulation (EFS).** EFS was used both in tracheal strip and perfused trachea preparations to examine the effect of $O_3$ on contractions and relaxations elicited by endogenous neurotransmitters. Strips were placed between two platinum ring electrodes situated at either end. Contractile and relaxation responses were obtained in some preparations under resting, spontaneous tone conditions and in other preparations after having obtained a stable contractile response to MCh ($3 \times 10^{-7}$ M). When perfused tracheae were used for EFS experiments, two platinum electrodes were aligned longitudinally on opposite sides of the mounted preparation. Both tracheal strip and perfused trachea preparations were stimulated electrically with 10-s trains of square-wave pulses (120 V, 0.5 ms) delivered at 7-min intervals to develop frequency-response curves. Responses to EFS were blocked in the presence of tetrodotoxin ($10^{-6}$ M; 30-min incubation; not shown), and contractions were antagonized by the muscarinic receptor blocker, atropine ($10^{-6}$ M; 30-min incubation; not shown). Other blockers were not used to isolate excitatory and inhibitory nonadrenergic, noncholinergic components of the responses (Fedan et al., 1986) for reasons that are explained in *Results*.

**Nitrotyrosine Immunofluorescence.** Tracheae and lungs were removed after perfusion of 10 ml of paraformaldehyde (4% w/v in 0.1 M PBS, pH 7.3) through the main pulmonary artery. Slices (5 mm) were placed into 4% (w/v) paraformaldehyde in PBS for 2 h, dehydrated in an increasing gradient of sucrose in PBS, embedded in a 1:1 solution containing OCT (Miles Inc., Elkhart, IN) and 20% sucrose in PBS, and frozen by 2-methylbutane in liquid nitrogen. Sections (5 $\mu$m) were cut and thaw-mounted onto precleaned slides. Nitrotyrosine immunostaining was performed according to the method of Ischiropoulos et al. (1995). The tissue was blocked with 4% BSA, 10% goat serum, and 0.3% Triton X-100 in 0.1 M PBS (pH 7.3) for 30 min. The tissue was washed with PBS and incubated with a polyclonal anti-nitrotyrosine antibody (Upstate Biotechnology, Lake Placid, NY) for 3 h, washed with PBS, and incubated for 1 h with anti-rabbit goat secondary antibody coupled to Texas Red. Tissues were prepared for immunofluorescence studies at 0, 6, and 18 h after exposure and were compared with air-exposed controls; the number of animals used to prepare replicate slides was three, three, four, and four for air control and 0, 6, and 18 h, respectively.

**Substance P Immunofluorescence and Assessment of Substance P Nerve Fiber Density.** The airways of the left lung were infused with picric acid-formaldehyde (Stefanini et al., 1967) fixative for 3 h and rinsed overnight in 0.1 M PBS containing 0.3% Triton X-100. The airways were microdissected and divided into axial bronchi (first and second order) and peripheral bronchi (third through sixth order) and frozen in 2-methylbutane isopentane cooled with liquid nitrogen. Cryostat sections (12 $\mu$m) of airways were cut and picked up on subbed slides. Immunocytochemical procedures for localizing substance P-immunoreactive neurons were identical to those described previously (Dey et al., 1990). Briefly, cryostat sections on coated coverslips were covered with rabbit anti-substance P antiserum (Peninsula, Belmont, CA) diluted 1:100, incubated in a humid chamber at 37°C for 30 min, rinsed with a 1% BSA-PBS buffer (pH 7.8) containing Triton-X solution, covered with fluorescein isothiocyanate-labeled goat anti-rabbit IgG (ICN Immunobiologicals, Inc., Costa Mesa, CA) diluted 1:100, incubated at 37°C for 30 min, rinsed again in BSA-PBS (pH 7.8) containing Triton-X solution, and mounted with Fluoromount G (Southern Biotechnology, Birmingham, AL). Controls for specificity of primary antiserum consisted of absorption of 1 mg/ml antiserum with substance P. Nonspecific background labeling was determined by omission of primary antiserum.

To assess substance P nerve fiber density, slides were blinded and scored for nerve fiber density in the epithelium, lamina propria, and smooth muscle by two readers using an arbitrary scoring index of 0 to +++ for least to greatest innervation density. Several (three to six) fields per slide were read on three separate slides from each animal for each tissue region of interest (epithelium, lamina propria, and smooth muscle), and the replicates of three slides for each animal were averaged; the values from both readers were then averaged for a final animal mean and a group mean ($n = 3$). Inasmuch as the tracheae of the animals were used for perfusion studies, in these preliminary experiments the epithelium, lamina propria, and smooth muscle of axial and peripheral bronchi of only the left lung were examined.

**Other Histological Examination.** Segments of trachea were fixed at in situ length in 10% phosphate-buffered formalin and embedded with Paraplast-Plus paraffin. Sections (5 $\mu$m) were stained with a routine Harris H&E procedure.

**MKH Solution.** MKH solution contained 113.0 mM NaCl, 4.8 mM KCl, 2.5 mM $CaCl_2$, 1.2 mM $KH_2PO_4$, 1.2 mM $MgSO_4$, 25.0 mM $NaHCO_3$, and 5.7 mM glucose, pH 7.4 (37°C), gassed with 95% $O_2$, 5% $CO_2$.

**General Protocol and Analysis of Results.** To reduce the effects of interanimal variability in the perfused trachea experiments (Fedan and Frazer, 1992) for each time point, tracheae from air- and $O_3$-exposed animals were examined at the same time. Likewise, to reduce the effects of interanimal variability on reactivity to inhaled MCh, air- and $O_3$-treated animals were examined at the same time. Differences in the ($\Delta P$) responses of perfused tracheae were noted and were due to differences in the initial size of the tracheae and the fifth-power relationship between radius and $\Delta P$ (Munakata et al., 1989), with smaller animals having smaller diameter tracheae and bigger $\Delta P$ responses and larger animals with larger diameter tracheae giving smaller $\Delta P$ responses (Fedan and Frazer, 1992).

Geometric mean $EC_{50}$ values were derived from least-squares analysis of a four-parameter logit curve fit and are presented with 95% confidence interval values in parentheses. Statistical comparisons of $EC_{50}$ values were made using normally distributed $-\log EC_{50}$ values. To analyze reactivity of the animals to inhaled MCh, the concentration required to produce a Penh response of 2 ($[MCh]_{Penh\text{-}2}$) was estimated in each animal by graphical interpolation from the dose-response curve. Statistical comparisons of these data were done using $-\log([MCh]_{Penh\text{-}2})$ values.

The results were analyzed for differences using repeated measures ANOVA, ANOVA on ranks, or Student's $t$ test for paired or nonpaired samples, as appropriate. Other results are expressed as mean ± S.E. unless otherwise stated; $n$ is the number of separate experiments. $P < .05$ was considered significant.

## Results

**Effects of $O_3$ Treatment on Reactivity to Inhaled MCh.** Immediately after the end of exposure (0 h), the basal Penh value of the $O_3$-treated animals was significantly increased (controls, 0.46 ± 0.08; $O_3$-treated, 0.91 ± 0.18). This was accompanied by a significant 3.88-fold leftward shift of the MCh dose-response curve and an increase in $-\log[MCh]_{Penh\text{-}2}$ (i.e., airway hyperreactivity to MCh; Fig. 1). Basal Penh and reactivity to inhaled MCh returned to the control level by 18 to 24 h after exposure. In animals exposed

to filtered air, basal Penh values were not changed by the exposure. In these animals, there were no changes observed except for a slight but significant increase at 24 h in the response to the 0.03-mg/ml MCh dose ($n$ = 6; data not shown); the $[MCh]_{Penh\text{-}2}$ values of these animals were not affected.

**Effects of $O_3$ Exposure In Vivo on Reactivity of Isolated, Perfused Trachea to MCh.** The results are shown in Fig. 2 and Table 1. As expected, reactivity to EL MCh was greater than IL reactivity. There were no effects of $O_3$ exposure on EL MCh concentration-response curves at any time point. In contrast, at 0 h after the end of exposure, the IL curve was shifted leftward by 5.4-fold, and the IL/EL maximum response ratio[2] was significantly increased compared with the air control. At 2 and 6 h after exposure, the size of the leftward shifts in the IL curves had diminished by 2.1- and 2.9-fold, respectively ($P < .161$ and $P < .055$, respectively). By 12 and 18 h, there were no effects of $O_3$ on IL MCh concentration-response curves.

**Epithelium Dependence of Increased Reactivity to IL MCh.** The lack of effect of $O_3$ on the EL MCh concentration-response curves suggested that IL hyperreactivity to MCh was epithelium-dependent. Figure 3 and Table 2 illustrate that in the absence of the epithelium, the IL MCh concentration-response curves of both air- and $O_3$-exposed tracheae were shifted to the left and the IL maximum responses were no longer different from the EL maximum responses (compare with Table 1). Furthermore, there were no effects of $O_3$ on the IL MCh concentration-response curves in the epithelium-denuded tracheae.

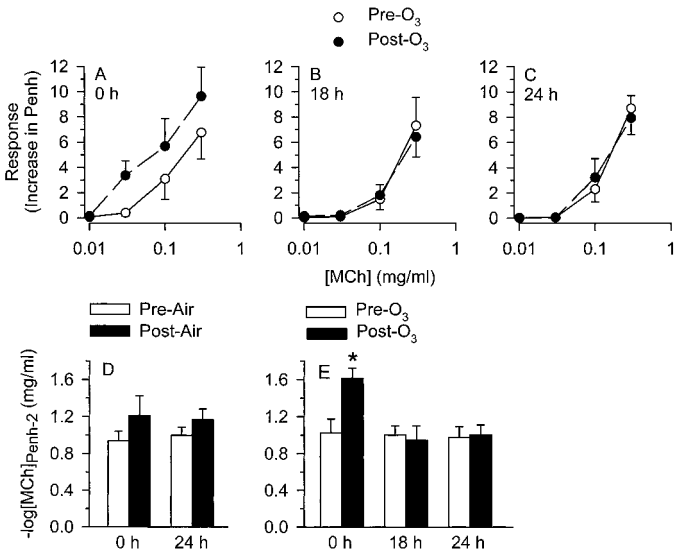


**Fig. 1.** Effect of $O_3$ treatment on reactivity of guinea pigs to inhaled MCh aerosol. Top row, dose-response curves obtained from animals examined 0 h (A), 18 h (B), and 24 h (C) after exposure to $O_3$. Each animal served as its own control, and different animals are depicted in each panel; the two curves in each panel show reactivity before ($PreO_3$) and after ($PostO_3$) $O_3$ treatment ($n$ = 6, 8, and 6 for 0, 18, and 24 h, respectively). The curves for air-treated controls are not shown. Bottom row, reactivity to MCh before and after air exposure (D; $n$ = 6 and 7 for 0 and 24 h, respectively) or before and after $O_3$ exposure (E). Sensitivity to MCh is expressed as the negative logarithm of the concentration of MCh that caused an increase in the value of Penh to 2 ($-\log[MCh]_{Penh\text{-}2}$), the individual values of which were interpolated from dose-response curves from each animal (an increase in the value of $-\log[MCh]$ signifies a decrease in $[MCh]_{Penh\text{-}2}$). *, significantly greater than $PreO_3$, signifying an increase in sensitivity to MCh. Air treatment had no effect on sensitivity to MCh.

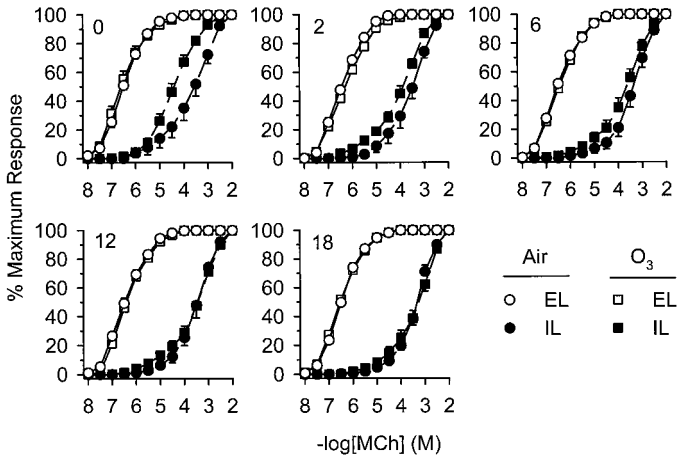


**Fig. 2.** Effect of $O_3$ exposure on MCh concentration-response curves of perfused tracheae. EL and IL concentration-response curves were obtained from tracheae removed from air- or $O_3$-exposed animals ($n$ = 5–8) immediately after (0 h) and 2, 6, 12, and 18 h after the end of exposure.

**Effect of $O_3$ Treatment on Relaxation Responses to IL Hyperosmolarity.** To examine the possibility that the increase in reactivity of perfused trachea to intraluminally applied MCh after $O_3$ treatment could involve a decrease in EpDRF release, NaCl was added to the IL MKH solution to evoke EpDRF-mediated relaxation responses. Figure 4 and Table 3 demonstrate that the relaxation responses were not affected at 0 h but became inhibited significantly at 2 and 6 h after exposure. By 12 h, the responses returned to the control level. $EC_{50}$ values for NaCl were not affected at any postexposure examination period (data not shown).

**Effect of $O_3$ Treatment on Neurogenic Responses of Tracheal Strip and Perfused Trachea Preparations.** Frequency-response relationships were obtained for neurogenic contraction and relaxation responses of tracheal strips under resting force or contracted with MCh to induce tone. Experiments on strips were included in this study because these preparations develop spontaneous tone, which allows visualization of relaxation responses without using an agent (e.g., MCh) that, although inducing contraction, could also affect the release of neurotransmitters. Figure 5 illustrates that there were no effects of $O_3$ on these responses 0 h after exposure. It was noted that EFS relaxed the strips beyond the level of force that had been induced with MCh; this is a reflection of the prostanoid-mediated, spontaneous tone (Orehek et al., 1975). In strips prepared from the same tracheae and run in parallel, there were no effects of $O_3$ on frequency-response curves for relaxation of uncontracted and MCh-contracted strips ($n$ = 14; data not shown).

To test the possibility that the lack of effect of $O_3$ on EFS-induced responses could have been due to the manipulations needed to prepare strips and the presence of spontaneous tone, we repeated the experiments using the perfused trachea, a reasonably "intact" airway that does not develop appreciable spontaneous tone. As shown in Fig. 6, $O_3$ treat-

[2] The IL/EL maximum response ratio is an index of the modulatory effect of the epithelium on the maximum contractile responses (Fedan and Frazer, 1992). The ratio is unity in the absence of epithelium and less than unity in the presence of the epithelium. The greater the inhibitory effect of the epithelium, the smaller is the value of the ratio.

TABLE 1
Effect of exposure to 3 ppm $O_3$ for 1 h on reactivity to extraluminal and intraluminal MCh in the perfused trachea

| Treatment ($n$) | $EC_{50}$ (95% CI) | | Maximum Response | | IL/EL Maximum Response Ratio |
|---|---|---|---|---|---|
| | EL | IL | EL | IL | |
| | *M* | | *cm $H_2O$* | | |
| 0 h | | | | | |
| Air (8) | $2.6 \times 10^{-7}$ (1.8–3.8) | $2.0 \times 10^{-4a}$ (0.4–8.9) | 28.9 ± 4.0 | 16.8 ± 3.4[b] | 0.58 ± 0.06 |
| $O_3$ (8) | $1.7 \times 10^{-7}$ (0.9–3.4) | $3.7 \times 10^{-5a,c}$ (1.8–7.7) | 16.9 ± 4.8 | 13.6 ± 4.2[b] | 0.72 ± 0.05[d] |
| 2 h | | | | | |
| Air (5) | $2.5 \times 10^{-7}$ (1.5–4.2) | $2.7 \times 10^{-4a}$ (0.9–8.3) | 13.0 ± 3.3 | 8.5 ± 1.6[b] | 0.71 ± 0.07 |
| $O_3$ (5) | $3.2 \times 10^{-7}$ (2.0–5.3) | $1.3 \times 10^{-4a}$ (0.6–2.9) | 13.3 ± 2.2 | 9.5 ± 1.6[b] | 0.71 ± 0.01 |
| 6 h | | | | | |
| Air (5) | $2.0 \times 10^{-7}$ (1.2–3.3) | $4.3 \times 10^{-4a}$ (1.6–11.4) | 13.2 ± 2.2 | 8.9 ± 1.5[b] | 0.66 ± 0.03 |
| $O_3$ (5) | $2.5 \times 10^{-7}$ (1.7–3.6) | $1.5 \times 10^{-4a}$ (0.7–3.2) | 14.5 ± 2.2 | 8.6 ± 1.7[b] | 0.58 ± 0.04 |
| 12 h | | | | | |
| Air (6) | $2.6 \times 10^{-7}$ (1.6–4.0) | $3.2 \times 10^{-4a}$ (2.0–5.4) | 9.3 ± 1.6 | 6.0 ± 1.0[b] | 0.67 ± 0.04 |
| $O_3$ (6) | $2.9 \times 10^{-7}$ (2.3–3.8) | $3.8 \times 10^{-4a}$ (2.5–5.7) | 16.2 ± 2.7 | 8.9 ± 1.6[b] | 0.54 ± 0.21[e,f] |
| 18 h | | | | | |
| Air (7) | $2.7 \times 10^{-7}$ (2.2–3.4) | $8.0 \times 10^{-4a}$ (4.7–13.7) | 14.0 ± 1.6 | 8.4 ± 0.9[b] | 0.60 ± 0.03 |
| $O_3$ (7) | $2.2 \times 10^{-7}$ (1.8–2.8) | $7.4 \times 10^{-4a}$ (2.1–2.6) | 10.8 ± 1.7 | 5.8 ± 0.8[b] | 0.55 ± 0.04[e] |

[a] Significantly larger than EL.
[b] Significantly less than EL. There were no differences in the IL/EL ratios between the air-treated controls.
[c] Significantly smaller than air.
[d] Significantly larger than air.
[e] Significantly less than 0 and 2 h.
[f] Significantly less than air.

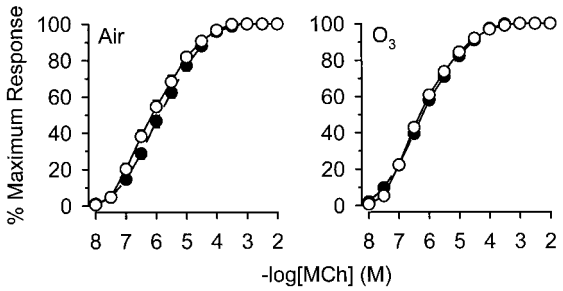


**Fig. 3.** Lack of effect of $O_3$ exposure on MCh concentration-response curves in epithelium-denuded, perfused tracheae. EL (○) and IL (●) concentration-response curves were obtained from tracheae removed from air- or $O_3$-exposed animals ($n$ = 6) 0 h after the end of exposure.

ment again had no effect on the responses. Because there was no effect, experiments were not conducted with MCh-contracted perfused tracheae to elicit EFS-induced relaxation responses.

**Effects of $O_3$ Exposure on Epithelial Bioelectric Properties.** Measurements of $V_T$ were made at 0 and 18 h after exposure to $O_3$. The 0-h postexposure time was chosen because it was the time of the greatest changes in in vivo and in vitro reactivity to MCh, whereas these changes had been reversed by 18 h, as described earlier. Figure 7 illustrates that $V_T$ was decreased at 0 h after exposure; at 18 h, $V_T$ was not different from the control value.

**Histological Examination of Airways.** Microscopic assessment of $O_3$-exposed tracheae detected several time-dependent alterations (Fig. 8). The air-exposed tracheal epithelium was characterized by the presence of ciliated cells, secretory cells, and basal cells (Fig. 8, A and H). Small to moderate numbers of inflammatory cells, principally neutrophils and eosinophils, infiltrated the epithelium of air-exposed tracheae. Despite variations in numbers, inflammatory cells of control guinea pigs consisted of discrete infiltrating cells, never cellular aggregates. Epithelial cells were arranged in a pseudostratified columnar pattern. Minimal variations in cilia and mucosubstances were occasionally observed, but only 1 of 34 air-exposed tracheae had moderate decreases in cilia and mucosubstances.

At 0 to 2 h after ozone exposure (Fig. 8, B and C), the epithelium was usually attenuated and frequently disorganized, contained decreased quantities of intraepithelial mucosubstances, and displayed fewer cilia. The lumen of many of these tracheae contained remnants of epithelium-appearing cells. These cells were characterized by eosinophilic cytoplasm, pyknotic nuclei, and cilia. At 6 h after exposure (Fig. 8D), cellular remnants were rare in the lumen, but decreases in cilia were consistent; most tracheae had decreased intraepithelial mucosubstances, and rare aggregates of intraepithelial eosinophils or neutrophils were seen in four of eight of these tracheae. At 12 and 18 h after exposure (Fig. 8, E and F), cellular remnants were not seen in the lumen, cilia and intraepithelial mucosubstances were decreased, and epithelial cells were mildly disorganized. Similar changes were seen 24 h after the end of the exposure (Fig. 8G).

We examined the appearance of nitrotyrosine adducts as a marker for elevated nitric oxide production in the airways. There was very little nitrotyrosine immunofluorescence in tracheal and lung sections of air-exposed animals (Figs. 9 and 10). However, 0 h after $O_3$ exposure, immunofluorescence was robust in detached tracheal epithelial cell ghosts

TABLE 2

Effect of exposure to 3 ppm $O_3$ for 1 h on reactivity to extraluminal and intraluminal MCh in epithelium-denuded perfused trachea

The tracheas were removed from the animals at 0 h postexposure.

| Treatment ($n$) | $EC_{50}$ (95% CI) | | Maximum Response | | IL/EL Maximum Response Ratio |
|---|---|---|---|---|---|
| | EL | IL | EL | IL | |
| | *M* | | *cm $H_2O$* | | |
| Air (6) | $3.9 \times 10^{-7}$ (1.7–9.2) | $9.0 \times 10^{-7}$ (4.8–18.6) | 10.5 ± 2.6 | 11.1 ± 2.7 | 1.06 ± 0.05 |
| $O_3$ (6) | $1.9 \times 10^{-7}$ (0.6–6.5) | $2.1 \times 10^{-7}$ (0.3–15.0) | 12.4 ± 2.9 | 12.4 ± 3.0 | 0.99 ± 0.06 |

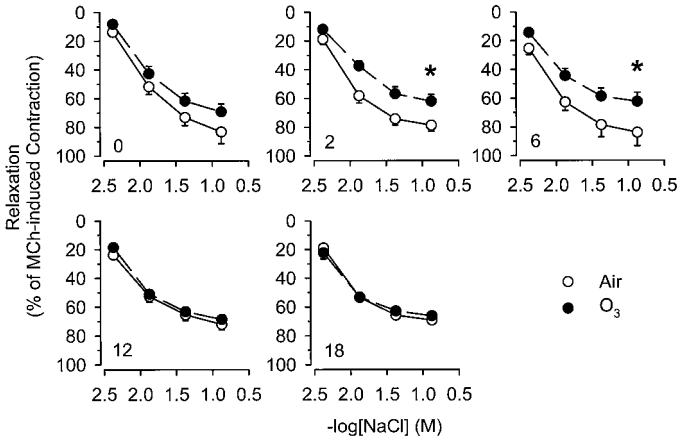


**Fig. 4.** Effect of $O_3$ exposure on EpDRF-mediated relaxation responses elicited with IL MKH solution made hyperosmolar with added NaCl. The preparations were contracted with EL MCh ($3 \times 10^{-7}$ M) before NaCl was added cumulatively. The curves were obtained from tracheae removed from air- or $O_3$-exposed animals ($n$ = 5–8) immediately after (0 h) or 2, 6, 12, and 18 h after the end of exposure. The abscissa indicates the molar concentrations of added NaCl. For NaCl, osmolarity is twice molarity. The osmolarity of the MKH solution is ~281 mOsM. *, significantly less than air-exposed controls.

TABLE 3

Effect of exposure to 3 ppm $O_3$ for 1 h on relaxation to intraluminal NaCl in the perfused trachea

| Treatment ($n$) | $EC_{50}$ (95% CI) | Maximum Response[b] |
|---|---|---|
| | *mM*[a] | *% MCh* |
| 0 h | | |
| Air (8) | 10.8 (8.9–13.2) | 83.2 ± 8.1 |
| $O_3$ (8) | 10.9 (8.8–13.5) | 69.1 ± 5.5[c] |
| 2 h | | |
| Air (5) | 7.7 (5.5–10.9) | 78.9 ± 4.7 |
| $O_3$ (5) | 11.2 (9.1–13.8) | 62.0 ± 4.2[c] |
| 6 h | | |
| Air (5) | 6.5 (3.9–11.0) | 85.2 ± 8.7 |
| $O_3$ (5) | 11.7 (5.6–24.7) | 62.6 ± 6.2[c] |
| 12 h | | |
| Air (6) | 7.5 (5.1–11.0) | 72.0 ± 3.9 |
| $O_3$ (6) | 7.1 (6.1–8.3) | 68.6 ± 3.2 |
| 18 h | | |
| Air (7) | 5.6 (3.9–8.1) | 66.2 ± 2.7 |
| $O_3$ (7) | 7.4 (5.8–9.5) | 69.2 ± 2.8 |

[a] Values refer to NaCl millimolar concentration added to MKH solution.
[b] Relaxation responses in centimeters of $H_2O$ normalized as a percentage of the extraluminal MCh ($3 \times 10^{-7}$ M)-induced contraction.
[c] Significantly less than air-treated controls.

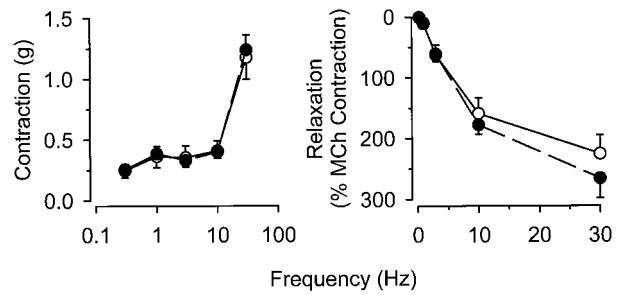


**Fig. 5.** Lack of effect of $O_3$ exposure on contractile responses (left) and relaxant responses (right) of tracheal strips to EFS. The results show contractions of preparations at resting tone (left) and relaxations of preparations that were first contracted with MCh ($3 \times 10^{-7}$ M; right). The strips were prepared from tracheae that were removed at 0 h after exposure [air (○) and $O_3$ (●) exposed, $n$ = 14].

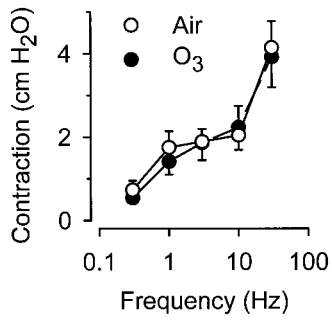


**Fig. 6.** Lack of effect of $O_3$ exposure on neurogenic contractile responses of perfused trachea. The tracheae were removed from the animals at 0 h after exposure (air- and $O_3$-exposed, $n$ = 8).

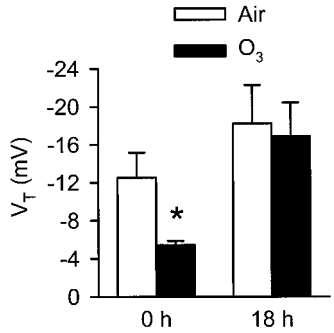


**Fig. 7.** Effect of $O_3$ exposure on $V_T$ of perfused trachea. *, significantly less than air treated.

(compare with Fig. 8). Elsewhere in the trachea, there were no changes in the level or pattern of immunofluorescence (Fig. 9). In lung sections, there was no discernible change in immunofluorescence at 0 h, but a strong signal appeared by 6 h and lasted for at least 18 h (Fig. 10). The location of these

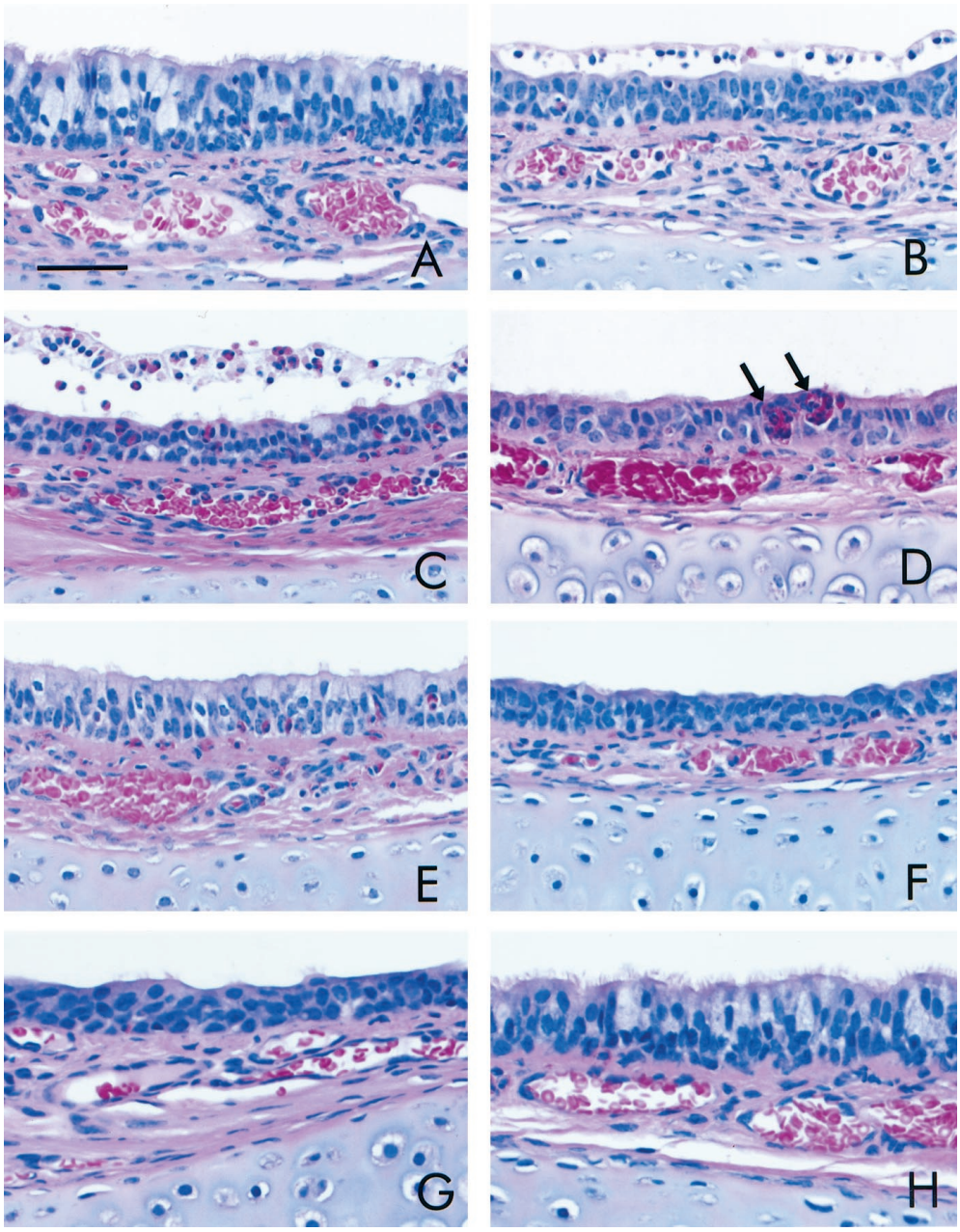


**Fig. 8.** H&E-stained histological sections (40×) of tracheae from air- and $O_3$-exposed guinea pigs. Tracheae were removed from animals after exposure to air (A, 0 h; H, 24 h) or $O_3$ (hours after exposure): 0 (B), 2 (C), 6 (D), 12 (E), 18 (F), or 24 (G). Arrows: aggregates of PMNs. Bar, 50 $\mu$m. These results are representative of at least four experiments at each time point.

cells primarily in lamina propria suggests that they were inflammatory cells (Pendino et al., 1995).

The greatest substance P nerve fiber density was observed in smooth muscle layers of the bronchi, exceeding that in the epithelium and lamina propria (Fig. 11). After $O_3$ exposure there were no changes in the densities of these fibers in epithelium. In axial bronchus, substance P fiber density was increased at 0 h in lamina propria and at 0 and 18 h in the smooth muscle. An increase in fiber density also was observed in the smooth muscle of peripheral airways at 0 h.

## Discussion

Our results suggest that the increase in reactivity to inhaled MCh after $O_3$ exposure involved greater accessibility of the drug through the mucosa to the smooth muscle and a decrease in the availability of EpDRF. The return of reactivity to the normal level by 18 to 24 h may have involved a reversal of these changes and is of particular interest because it occurred concurrently with substantial alterations in mucosal morphology.

Immediately after $O_3$ exposure, animals became hyperreactive to inhaled MCh, the perfused trachea was more sensitive to IL MCh, the IL/EL maximum response ratio was increased, and $V_T$ was decreased. EpDRF-mediated relaxation was not inhibited immediately but became significantly inhibited at 2 h. The increased reactivity to IL MCh was not observed in tracheae devoid of the epithelium. This finding indicates that the epithelium was the site of the effects of $O_3$,

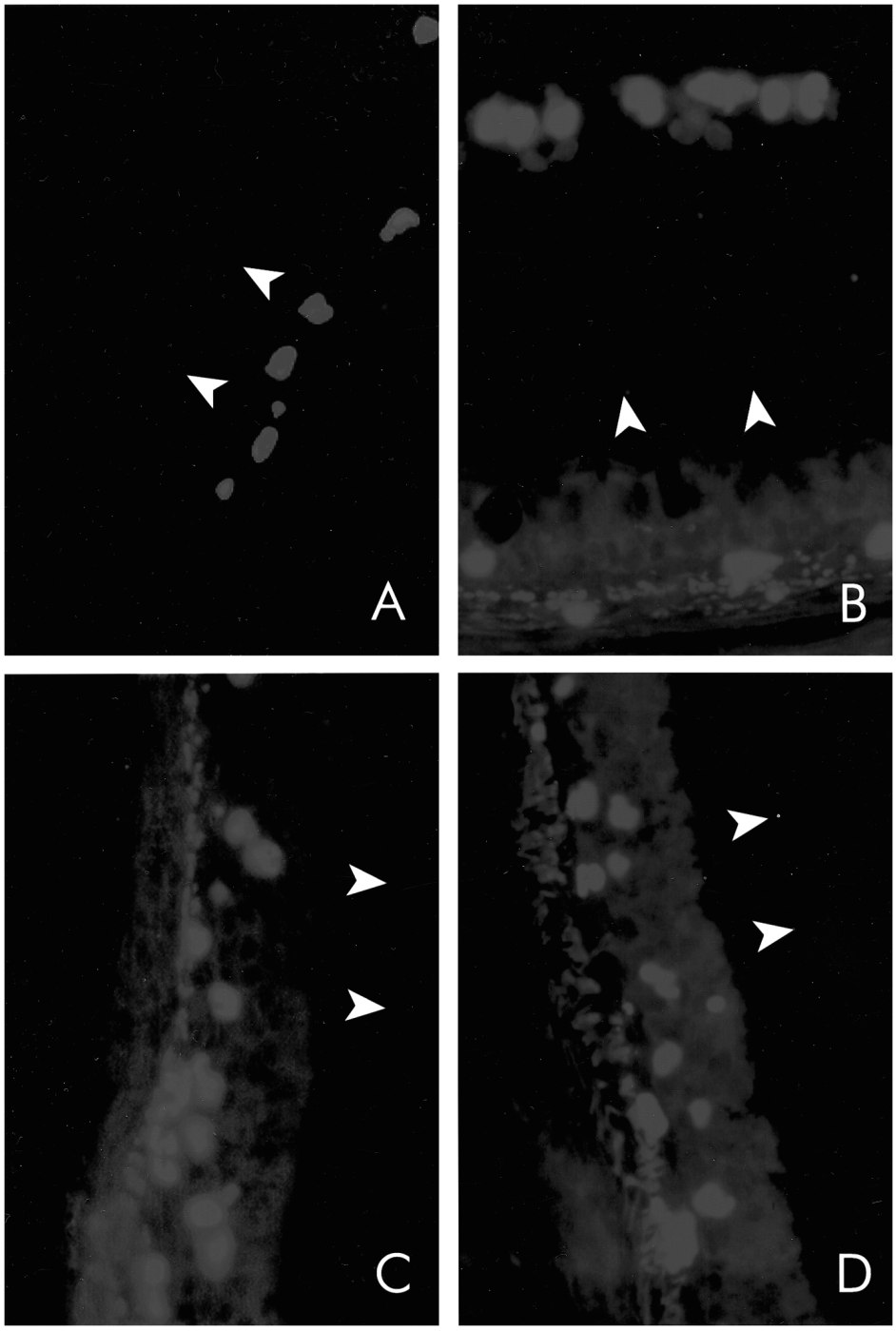


**Fig. 9.** Effect of $O_3$ exposure on nitrotyrosine immunofluorescence in trachea. A, control; B, 0 h; C, 6 h; D, 18 h. Note by comparison with Fig. 8 that detached cell ghosts were highly stained at 0 h. Immunostaining was also present in the mucosa of the controls, but there were no changes in these areas. Arrows point toward the luminal side of the trachea. These results are representative of at least three experiments at each time point.

not the muscle, which is in agreement with previous observations (Montaño et al., 1993; Roux et al., 1996). By 18 to 24 h after exposure, reactivity to inhaled and IL MCh and reactivity to IL NaCl returned to the control levels. The changes in IL reactivity to MCh and NaCl, except for the lag in the development of hyporeactivity to NaCl, followed comparable time courses, were greatest 0 h after exposure, and became progressively attenuated and returned to control over a 12-h postexposure period. The greatest increase in in vivo and in vitro reactivity to MCh, occurring at 0 h after exposure, could not have been due to reduced EpDRF production or effect.

We did not observe an effect of $O_3$ on neurogenic responses of strip or perfused trachea preparations. Neurogenic contractile responses in the guinea pig trachea involve cholinergic and noncholinergic excitatory efferents, and relaxant responses are mediated by nitrergic and, possibly, neuropeptidergic nerves (Tucker et al., 1990; Undem et al., 1996). Substance P fiber density was elevated in airway smooth

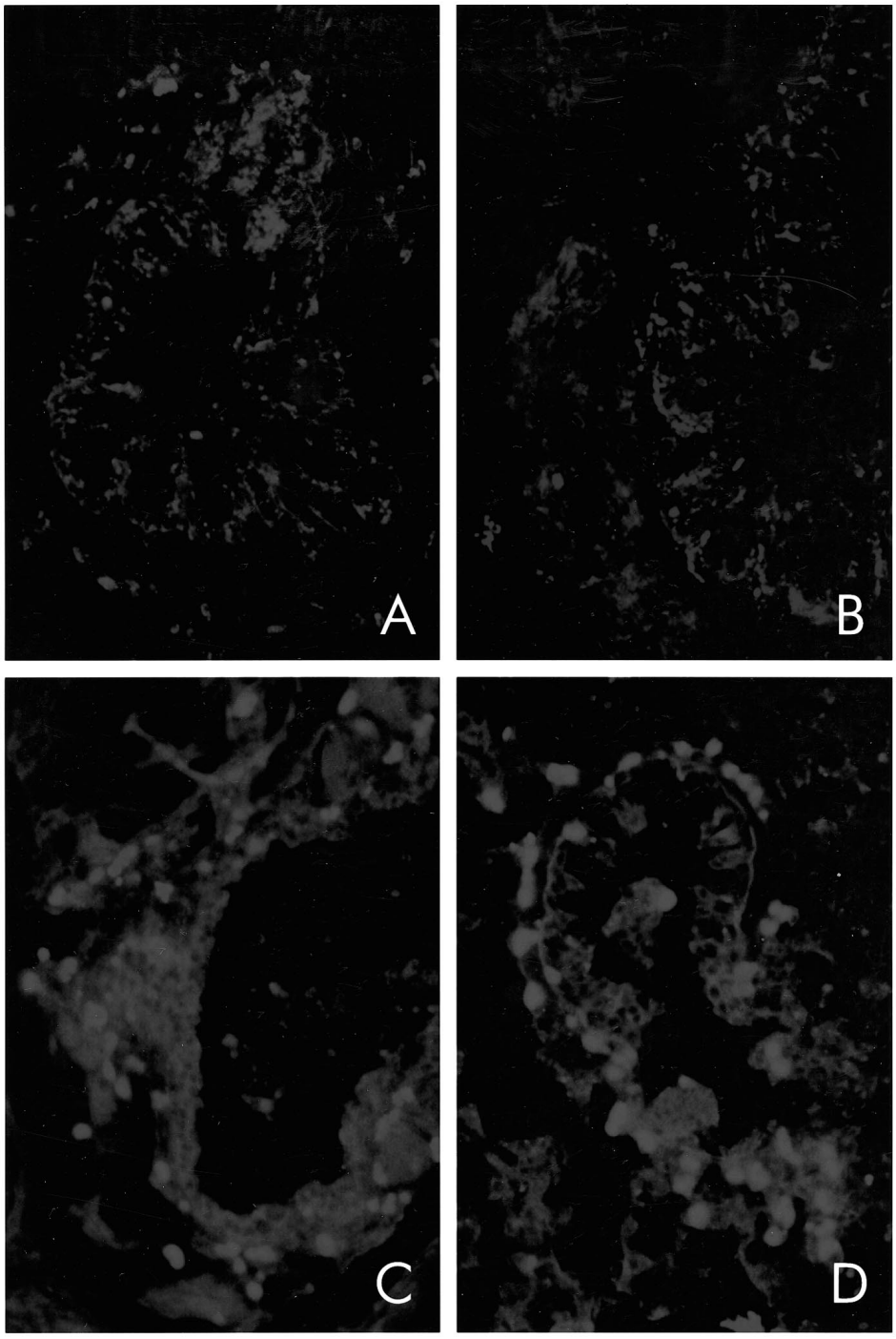


**Fig. 10.** Effect of $O_3$ exposure on nitrotyrosine immunofluorescence in lung. A, control; B, 0 h; C, 6 h; D, 18 h. Note that immunostaining in the wall of the airways was not elevated at 0 h after $O_3$ exposure but appeared by 6 h. These results are representative of at least three experiments at each time point.

muscle by $O_3$ treatment, but the neurogenic responses in the trachea did not reflect up-regulation of this potent contractile peptide. $O_3$ potentiated in vivo the obstructive response of guinea pigs to vagal stimulation, an effect that involved the disruption of prejunctional $M_2$ muscarinic autoreceptors (Costello et al., 1998). Our present in vitro experiments do not suggest that disruption of $M_2$ receptors on postganglionic, cholinergic nerve fibers had occurred, inasmuch as responses of perfused tracheae or tracheal strips to EFS were not altered after $O_3$ treatment. Sommer et al. (1997) also observed no effect of $O_3$ treatment (1.2 ppm, 4 h), 16 to 18 h after exposure, on EFS-induced contractile responses of in vitro guinea pig tracheal preparations.

Neurogenic contractile and relaxant responses and the release of transmitters are modulated by the epithelium (e.g., epithelium removal in vitro potentiates EFS-induced cholinergic contractile responses, whereas EpDRF inhibits them; Flavahan et al., 1985; Takata et al., 1995; Fedan et al., 1999). Therefore, the lack of effect of $O_3$ on EFS-induced responses raises the following question: if the epithelium modulates

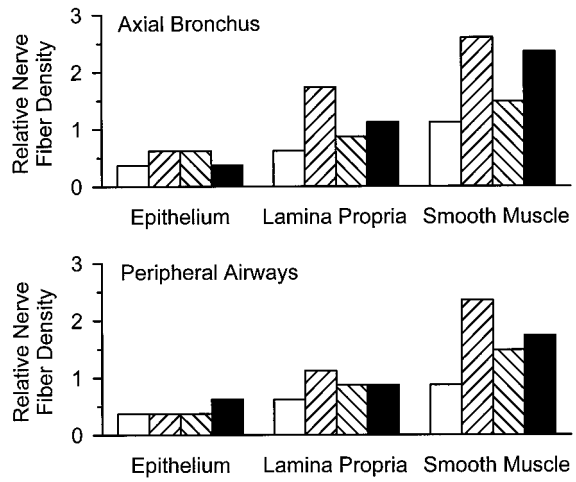


**Fig. 11.** Effect of $O_3$ exposure on substance P immunofluorescence in epithelium, lamina propria, and smooth muscle in extrapulmonary (top) and intrapulmonary (bottom) bronchi. The ordinate values represent substance P-containing nerve fiber density using an arbitrary scale of 0 to +++. Values are the averages of the triplicate measurements of blinded slides made independently by two readers. □, air (0 h); ▨, $O_3$ (0 h); ▧, air (18 h); ■, $O_3$ (18 h).

neurotransmission, and the epithelium is damaged sufficiently by $O_3$ to increase reactivity to MCh and disrupt EpDRF-mediated relaxations, why are the neurogenic responses not altered? This finding implies that EpDRF modulation of efferent neural function was not changed by $O_3$. Previous evidence for epithelial modulation of neurotransmission was derived from experiments involving complete epithelium removal or activation of EpDRF release from healthy epithelium. In the present study, $O_3$ disrupted the function of but did not entirely denude the epithelium. Experiments comparing the effects of epithelium removal on neurogenic responses of control and $O_3$-treated animals will be needed to understand whether the remaining cells retained neuromodulatory capability.

$V_T$ was depolarized 0 h after $O_3$ and returned to the control level by 18 h, in parallel with the increase in reactivity to inhaled and IL MCh and their return to normal levels, and nearly in parallel with the decrease and increase in responsiveness to IL NaCl. Different effects were reported by Stutts and Bromberg (1987), who observed after the exposure of guinea pigs to $O_3$ (1 ppm, 3 h) an increase in $V_T$ 3 h after the end of exposure without a change in mucosal permeability to mannitol. By 24 h after exposure, the potential difference had decreased and mannitol permeability had increased. More recently, Croxton et al. (1994) observed that $O_3$ (1 and 2 ppm; 3 h) had no effect on potential difference in isolated guinea pig trachea 0 and 6 h after exposure. Their in vivo measurements in treated mice (2 ppm, 3 h), however, did reveal an epithelial depolarization and an increase in paracellular conductance (Takahashi et al., 1993, 1995). In rabbits, $O_3$ treatment (0.2 ppm, 7 h) resulted 3 h later in epithelial depolarization (Freed et al., 1996). Thus, our findings agree with previous results of mouse and rabbit experiments but not those with guinea pigs. The differences among the guinea pig results could be due to differences in the sizes of the animals or to the exposure protocols used. For example, a 3-h exposure period was used previously, and the earliest measurements were made at the end of the exposure (Stutts and Bromberg, 1987; Croxton et al., 1994). It is possible that the depolarization we observed is an immediate and transient event that had waned by 3 h. The mechanism of the bioelectric changes caused by $O_3$ is unknown at present and could involve alterations in the paracellular pathway and transepithelial resistance (Takahashi et al., 1993, 1995) and/or cellular transport mechanisms, which could not be discriminated with the apparatus used in the current experiments.

No correlation between the inflammatory response in the airway wall and the onset of hyperreactivity to MCh in vivo or in vitro was evident in our study. In fact, the onset of the inflammatory response began after the increase in reactivity to MCh had peaked. Similar findings were reported by Schultheis and Bassett (1994) after $O_3$ exposure (2 ppm, 4 h) of guinea pigs. Schultheis and Bassett also noted that PMNs remained in the lavage fluid for several days, and Matsubara et al. (1995) observed elevated neutrophils in lavage fluid after hyperreactivity had been reversed, 5 to 24 h after exposure. Our findings indicate that inflammation is unlikely to have initiated the hyperreactivity, which is in agreement with the results seen in previous investigations (Evans et al., 1988; Kleeberger and Hudak, 1992; Young and Bhalla, 1992; Reinhart et al., 1998). However, inhibition of EpDRF-mediated relaxation responses to NaCl occurred not immediately but after a 2-h delay, corresponding with clear influx of inflammatory cells.

We sought evidence for possible alterations in nitric oxide production after $O_3$ treatment using the appearance of nitrotyrosine as a marker. In the trachea, nitrotyrosine immunofluorescence was increased at 0 h only in epithelial cell ghosts that had been detached. We do not know whether this increase reflective of nitric oxide synthase activation was an initiating event in the epithelial damage caused by $O_3$. The lack of an increase in the remaining cells suggests that nitric oxide was not involved in the increase in reactivity to MCh, at least in the isolated trachea. In lung sections, nitrotyrosine immunofluorescence was not evident at 0 h but was evident in bronchi at 6 h. It is possible that the increase in the production of this bronchodilator in the smaller airways contributed to the reversal of reactivity to inhaled MCh. Clearly, a regional difference exists in the stimulation of nitrotyrosine formation after $O_3$ exposure. These results also suggest indirectly that nitric oxide is not EpDRF (Munakata et al., 1990; Fedan et al., 1999).

Our findings agree with previous studies (3 ppm, 2 h) using tracheal rings and strips prepared from $O_3$-exposed guinea pigs in which it was concluded that the epithelium primarily was involved in the increase in responsiveness to muscarinic agonists (Murlas et al., 1990; van Hoof et al., 1997), at least in vitro. The epithelium requirement for altered mucosal reactivity to MCh in the perfused trachea is a complementary observation to previous findings in guinea pigs (Yeadon et al., 1992; Matsubara et al., 1995) in which a similar $O_3$ exposure protocol (3 ppm, up to 2 h) led to hyperreactivity of the animals to inhaled MCh but not to intravenously administered MCh. Matsubara et al. (1995) also observed that hyperreactivity to inhaled MCh was not evident after 24 h. Campos et al. (1992) found that $O_3$ (3 ppm, 1 h) had no effect on responsiveness of tracheal chains to histamine at 16 to 18 h after exposure, but reactivity to substance P in tracheal chains was increased.

The significance of our findings extends beyond the effects of $O_3$ per se. First, the results indicate that the mechanisms

involved in the modulation of reactivity in vivo and in vitro by the epithelium can be normal even when the mucosa is damaged and remodeled. Second, EpDRF can be produced by one or more of the cell types that were not detached; whether the sloughed cells have this capability is not known. Last, the return of $V_T$ to normal values suggests that epithelial bioelectric properties returned concurrent with reestablishment of normal barrier function and EpDRF production.

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