Capsaicin-sensitive C-fiber-mediated protective responses in ozone inhalation in rats

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Capsaicin-sensitive C-fiber-mediated protective responses in ozone inhalation in rats. J. Appl. Physiol. 86(3): 951–962, 1999.—To assess the role of lung sensory C fibers during and after inhalation of 1 part/million ozone for 8 h, we compared breathing pattern responses and epithelial injury-inflammation-repair in rats depleted of C fibers by systemic administration of capsaicin as neonates and in vehicle-treated control animals. Capsaicin-treated rats did not develop ozone-induced rapid, shallow breathing. Capsaicin-treated rats showed more severe necrosis in the nasal cavity and greater inflammation throughout the respiratory tract than did control rats exposed to ozone. Incorporation of 5-bromo-2′-deoxyuridine (a marker of DNA synthesis associated with proliferation) into terminal bronchiolar epithelial cells was not significantly affected by capsaicin treatment in rats exposed to ozone. However, when normalized to the degree of epithelial necrosis present in each rat studied, there was less 5-bromo-2′-deoxyuridine labeling in the terminal bronchioles of capsaicin-treated rats. These observations suggest that the ozone-induced release of neuropeptides does not measurably contribute to airway inflammation but may play a role in modulating basal and reparative airway epithelial cell proliferation.

C fibers; ozone; 5-bromo-2′-deoxyuridine labeling; morphometry; rapid, shallow breathing

THE ACUTE INHALATION OF OZONE, a ubiquitous constituent of photochemical air pollution, has been shown to result in respiratory tract epithelial injury and inflammation in rats (14, 23) and human subjects (29). The ozone-induced lesion has been shown to involve the anterior nares, the ciliated epithelium of the trachea, large bronchi, terminal bronchioles, and the type 1 pneumocytes of the respiratory bronchioles and alveolar ducts. Possible factors that may be involved in determining this pattern of distribution of ozone-induced lesions include, but are not limited to, ozone’s relative water insolubility and high reactivity with components of airway-lining fluid, airway geometry, ventilatory pattern (minute ventilation (Ve), tidal volume (Vt), and breathing frequency (f)), and the regional sensitivity of airway epithelial cells to oxidant damage.

Data from a recently published study from our laboratory are consistent with the notion that airway sensory C fibers play an important modulatory role in ozone-induced injury and inflammation of the terminal bronchioles and alveolar ducts (30). In that study, Sterner-Kock and co-workers (30) demonstrated that adult rats chemically ablated of C fibers by neonatal capsaicin treatment had significantly more ozone-induced injury in small peripheral airways and pulmonary parenchyma than did normal rats exposed to ozone. Numerous sensory C-fiber-dependent mechanisms, acting through central reflexes and/or the release of neuropeptides within the airways, could be responsible for these observations.

Our overall hypothesis is that lung C fibers, via reflexes and/or local neuropeptide release, act to lessen the damaging effects of ozone in the respiratory tract. In the present study, we have further examined this hypothesis by assessing the severity and distribution of ozone-induced epithelial injury throughout the entire respiratory tract, including the nasal cavity, in rats ablated of sensory C fibers by neonatal capsaicin treatment. We used an experimental design that allowed us to relate observed alterations in the distribution and severity of ozone-induced lesions to measured changes in breathing pattern and tracheal substance P (SP) content. Finally, morphometric techniques measuring the incorporation of 5-bromo-2′-deoxyuridine (BrdU) into replicating epithelial cells within terminal bronchioles enabled us to assess the repair process in capsaicin-treated rats compared with control rats after ozone inhalation.

METHODS

Animals and capsaicin treatment. We obtained pregnant Wistar rats (~14 days gestation) from a specific pathogen-free colony (Charles River Laboratories, Wilmington, MA) and housed them individually in polycarbonate cages with fir wood chips. The animals were maintained on a 12:12-h light-dark photoperiod and provided Purina Rodent Laboratory Chow 5001 (Purina Mills, St. Louis, MO) and water ad libitum. We randomly divided the pregnant rats into two groups: capsaicin-treated animals and vehicle-treated control animals. Newborn male pups from the first group were treated on day 2 of life with a single subcutaneous injection of capsaicin (Sigma Chemical, St. Louis, MO) dissolved in olive oil (10 mg capsaicin/ml olive oil) at a dosage of 50 mg/kg body wt. Pups in the vehicle control group were treated with olive oil only. After treatment of the pups, we returned them to their mothers and weaned them at 21–28 days. Beginning ~1 wk before weaning, tetracycline (1 mg/ml) was added to the drinking water. We did this to minimize development of skin...
Fig. 1. Schematic of ozone (O₃)-exposure system. Design of system was as follows: 1) vehicle-treated control rats exposed to filtered air (FA) for 16 h (n = 10); 2) vehicle-treated control rats exposed to 1 part/million (ppm) ozone for 8 h followed by an 8-h postexposure period of FA (n = 10); 3) capsaicin-treated rats exposed to FA for 16 h (n = 10); and 4) capsaicin-treated rats exposed to 1 ppm ozone for 8 h followed by an 8-h postexposure period of FA (n = 9). Therefore, the experimental time for each rat was 16 h total.

Anesthesia and ozone inhalation. We anesthetized the rats with a solution of 2% α-chloralose, 25% urethan, and 5% sodium tetraborate (0.3 ml/100 g body wt ip, Sigma Chemical). We inserted a sterile catheter into the femoral vein of each rat and attached the catheter to an infusion pump. Then we maintained anesthesia with a constant infusion of 2% α-chloralose, 25% urethan, and 5% sodium tetraborate. We placed the rats on a water-filled heating pad and inserted a rectal temperature probe; body temperature was maintained at 37 ± 0.5°C.

We exposed rats to ozone (1 ppm) or FA by using a head-only inhalation system (Fig. 1). Briefly, medical-grade oxygen and FA were introduced into the system through a calibrated flow panel at known flow rates. Ozone was produced by passing oxygen through a Sanders model 25 Ozonizer (Dasibi Environmental, Glendale, CA). We inserted a sterile catheter into the femoral vein of each rat and attached the catheter to an infusion pump. Then we maintained anesthesia with a constant infusion of 2% α-chloralose, 25% urethan, and 5% sodium tetraborate.

During an experiment, we prevented gas flow through the exposure hoods. Before attaching the rats to the system, we balanced the pneumotachographs to zero voltage with full flow (1 l/min) going through them. The rats' VT, f, and V˙E were then measured by integrating the periodic change in flow passing through the pneumotachograph by using a PO-NE-MAH digital data-acquisition and -analysis system (PO-NE-MAH/Gould Instrument Systems, Valley View, OH). During the 16-h experimental period, VT, f, and V˙E were measured and recorded for 120 s every 20 min. The rats' VT were then measured by integrating the periodic change in flow passing through the pneumotachograph by using a PO-NE-MAH digital data-acquisition and -analysis system (PO-NE-MAH/Gould Instrument Systems, Valley View, OH). During the 16-h experimental period, VT, f, and V˙E were measured and recorded for 120 s every 20 min. The rats' VT were then measured by integrating the periodic change in flow passing through the pneumotachograph by using a PO-NE-MAH digital data-acquisition and -analysis system (PO-NE-MAH/Gould Instrument Systems, Valley View, OH). During the 16-h experimental period, VT, f, and V˙E were measured and recorded for 120 s every 20 min.

In vivo cumulative cell labeling. After placing the femoral vein catheter, we inserted one osmotic minipump (Alzet pump model 2ML1, nominal pumping rate 10 μl/h, Alza, Palo Alto, CA) subcutaneously between the shoulder blades. This pump, primed by immersion in 0.9% saline at 37°C for 8 h, was loaded with BrdU (Sigma Chemical) dissolved in PBS (20 mg/ml). BrdU, a thymidine analog, is incorporated into DNA by cells undergoing DNA replication and is indicative of cell proliferation and a marker of ozone-induced airway epithelial injury (25). The duodenum was used as a labeling control for each rat. Incorporated BrdU was detected by using a monoclonal antibody and immunocytochemical techniques as outlined by Hsu et al. (12, 13). Briefly, 30-µm-thick paraffin sections were deparaffinized, DNA was denatured by using 2.5 N HCl, and endogenous peroxidase activity was blocked by 3% hydrogen peroxide followed by 5% normal rabbit serum with 5% powdered milk added to block nonspecific binding. Sections were incubated with the primary monoclonal mouse anti-BrdU (Dako, Carpenteria, CA) diluted 1:50 in PBS with 5% powdered milk for 2 h. For negative control sections, we incubated tissue sections by using only PBS with 5% powdered milk. The sections were then washed with PBS and incubated 30 min in biotinylated, rabbit anti-mouse immunoglobulin (Dako) diluted 1:200 in PBS with 5% normal rabbit serum. We followed this procedure by incubation in Vectastain ABC reagent (Vector Laboratories, Burlingame, CA). Sections were incubated in peroxidase substrate solution (7.5 μl 30% hydrogen peroxide in 1.0 mg/ml diaminobenzidine in 0.1 M Tris·HCl buffer at pH 7.6) for 3–5 min. No counterstain was used, and labeled nuclei appeared reddish-brown.

Quantification of tracheal SP levels. We evaluated the effect of neonatal capsaicin treatment and ozone inhalation on airway SP by quantifying SP levels in tracheal homogenates. In brief, we obtained segments of trachea (~0.5 cm long) at the end of each experiment before fixing the lungs for morphometry. The tracheal segments were placed in 0.5 ml 2 N glacial acetic acid, rapidly frozen, and stored at −70°C. SP in tracheal segments was extracted and assayed by using a commercially available enzyme immunoassay kit (Cayman Chemical, Ann Arbor, MI) as previously described (50).

Intravascular perfusion fixation and tissue preparation. We fixed the lungs and nasal cavity by intravascular perfusion via the pulmonary artery and aorta, respectively. We chose...
this fixation for this study to avoid the redistribution of intraluminal inflammatory cells and sloughed epithelial cells in airways and alveoli (3). Briefly, we performed a tracheotomy on the anesthetized rats to ventilate the lungs with room air by using a small-animal respirator (60 breaths/min). The chest was opened, and 0.15 ml of heparin (1,000 units/ml) was injected into the right and left ventricles. We then cannulated the pulmonary artery and aorta. Both atria were then removed to allow the escape of fluid from the pulmonary and systemic circulation. The pulmonary vasculature was flushed with PBS until the lungs were white and then was perfused under pressure of ~18 mmHg for the lungs and 40 mmHg for the nasal cavity. After perfusion, lungs, heads, and duodenum (BrdU-positive control tissue) were stored in fixative for at least 48 h before processing.

We dissected the fixed lungs by using a dissecting microscope and a cool fiber-optic illuminator. The fixed right cranial lung lobe was dissected along its long axis, beginning at the level of the lobar bronchus. The plane through which we dissected the lobe exposed the majority of small side branches. The tissues were then processed and embedded in paraffin for light microscopy according to standard procedures. We cut a 7-µm-thick section from the anterior surfaces of the paraffin-embedded blocks and embedded in paraffin for light microscopy by using a semiquantitative scoring system without knowledge of exposure group (Table 1). Six-micrometer-thick sections were cut from the anterior surfaces of the paraffin-embedded blocks and stained with H&E.

Airway morphology and semiquantitative scoring system. We assessed the H&E sections of nasal cavities and lungs by light microscopy using a semiquantitative scoring system without knowledge of exposure group (Table 1). Semiquantitative scoring systems have been shown to provide a relatively reproducible assessment of pathological changes in the lung (4, 30). The scoring scheme in this study was modified from the scheme we used previously (30) to include the nasal cavity. Briefly, 17 histopathological features within the nasal cavity, bronchi, terminal bronchioles, and proximal alveolar ducts were divided into 2 broad sections, inflammatory and/or exudative changes and epithelial changes, and scored in a semiquantitative fashion. Scores were based on a 0–5 grading scheme, where 1 = absent, 2 = occasional, 3 = <25% tissue involvement, 4 = 25–50% tissue involvement, and 5 = >50% tissue involvement.

Morphometry of the lungs. We examined the 7-µm-thick H&E sections of lung under low magnification and numbered the terminal bronchioles by using National Institutes of Health Image 2.0 software (Bethesda, MD).

Table 1. Grading scheme scores for histopathological features in ozone-induced nasal and pulmonary lesions

<table>
<thead>
<tr>
<th></th>
<th>Vehicle-Treated Control Rats Exposed to FA (n = 10)</th>
<th>Vehicle-Treated Control Rats Exposed to O3 (n = 10)</th>
<th>Capsaicin-Treated Rats Exposed to FA (n = 10)</th>
<th>Capsaicin-Treated Rats Exposed to O3 (n = 9)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Inflammatory/exudative changes</strong></td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Nasal cavity</td>
<td></td>
<td></td>
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<tr>
<td>Inflammatory cells in lamina propria/submucosa, extent</td>
<td>2.20 ± 0.133</td>
<td>2.30 ± 0.153</td>
<td>2.20 ± 0.133</td>
<td>2.78 ± 0.278</td>
</tr>
<tr>
<td>Inflammatory cells in lamina propria/submucosa, severity</td>
<td>2.20 ± 0.133</td>
<td>2.20 ± 0.133</td>
<td>2.30 ± 0.153</td>
<td>2.78 ± 0.278†</td>
</tr>
<tr>
<td>Intraepithelial inflammatory cells</td>
<td>1.10 ± 0.100</td>
<td>1.90 ± 0.277*</td>
<td>1.20 ± 0.200</td>
<td>2.78 ± 0.364**</td>
</tr>
<tr>
<td>Luminal exudate</td>
<td>1.40 ± 0.221</td>
<td>2.50 ± 0.167*</td>
<td>1.90 ± 0.180</td>
<td>2.89 ± 0.309*</td>
</tr>
<tr>
<td>Bronchi</td>
<td></td>
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<tr>
<td>Intersitial inflammatory cells, extent</td>
<td>2.10 ± 0.100</td>
<td>2.20 ± 0.133</td>
<td>2.10 ± 0.100</td>
<td>2.56 ± 0.176*</td>
</tr>
<tr>
<td>Intersitial inflammatory cells, severity</td>
<td>2.20 ± 0.133</td>
<td>2.20 ± 0.133</td>
<td>2.20 ± 0.133</td>
<td>2.67 ± 0.167†</td>
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<tr>
<td>Terminal bronchioles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Inflammatory cells in bronchial wall, extent</td>
<td>1.00 ± 0.000</td>
<td>1.20 ± 0.133</td>
<td>1.00 ± 0.000</td>
<td>1.89 ± 0.261*†</td>
</tr>
<tr>
<td>Inflammatory cells in bronchial wall, severity</td>
<td>1.00 ± 0.000</td>
<td>1.20 ± 0.133</td>
<td>1.00 ± 0.000</td>
<td>1.67 ± 0.167*†</td>
</tr>
<tr>
<td>Mixed inflammatory cells in interstitium</td>
<td>2.20 ± 0.133</td>
<td>2.60 ± 0.163</td>
<td>2.20 ± 0.133</td>
<td>3.00 ± 0.236*</td>
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<tr>
<td>Proximal alveolar ducts</td>
<td></td>
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<tr>
<td>Inflammatory cells in interalveolar septa adjacent to centriacinar regions</td>
<td>2.10 ± 0.100</td>
<td>2.60 ± 0.163*</td>
<td>2.10 ± 0.100</td>
<td>3.00 ± 0.289*</td>
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<tr>
<td>Presence of alveolar macrophages</td>
<td>1.90 ± 0.100</td>
<td>2.00 ± 0.00</td>
<td>2.20 ± 0.133</td>
<td>2.33 ± 0.167</td>
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<tr>
<td><strong>Epithelial changes</strong></td>
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<td></td>
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<tr>
<td>Nasal cavity</td>
<td></td>
<td></td>
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<tr>
<td>Necrosis and sloughing of transitional epithelium, extent</td>
<td>1.30 ± 0.300</td>
<td>2.00 ± 0.211*</td>
<td>1.50 ± 0.342</td>
<td>2.89 ± 0.309†</td>
</tr>
<tr>
<td>Necrosis and sloughing of transitional epithelium, severity</td>
<td>1.20 ± 0.200</td>
<td>1.80 ± 0.133*</td>
<td>1.50 ± 0.342</td>
<td>3.00 ± 0.333*</td>
</tr>
<tr>
<td>Bronchi</td>
<td></td>
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</tr>
<tr>
<td>Vacuolation, necrosis and sloughing of epithelium, extent</td>
<td>1.00 ± 0.000</td>
<td>2.30 ± 0.335</td>
<td>1.00 ± 0.000</td>
<td>2.33 ± 0.167*</td>
</tr>
<tr>
<td>Vacuolation, necrosis and sloughing of epithelium, severity</td>
<td>1.00 ± 0.000</td>
<td>1.80 ± 0.200*</td>
<td>1.00 ± 0.000</td>
<td>2.33 ± 0.167*</td>
</tr>
<tr>
<td>Terminal bronchioles</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Vacuolation, necrosis and sloughing of epithelium, extent</td>
<td>1.00 ± 0.000</td>
<td>2.00 ± 0.258</td>
<td>1.00 ± 0.000</td>
<td>2.44 ± 0.176*</td>
</tr>
<tr>
<td>Vacuolation, necrosis and sloughing of epithelium, severity</td>
<td>1.00 ± 0.000</td>
<td>1.80 ± 0.200*</td>
<td>1.00 ± 0.000</td>
<td>2.33 ± 0.167*</td>
</tr>
</tbody>
</table>

Values are means ± SE. n, No. of rats. Scores are based on a 0–5 grading scheme, where grade 1 = absent, 2 = occasional, 3 = <25% tissue involvement, 4 = 25–50% tissue involvement, and 5 = >50% tissue involvement. *Significantly different (P < 0.05) from corresponding FA-exposed group. †Significantly different (P < 0.05) from capsaicin-treated rats exposed to O3.

Vacuolation, necrosis and sloughing of epithelium, severity
Health Image software.¹ Four terminal bronchioles were systematically sampled by using a random start from the 10–20 terminal bronchioles sectioned per slide. We counted BrdU-positive epithelial cells by using an optical “disector” (2). We chose terminal bronchioles because the primary lesion of acute ozone toxicity in the lung is epithelial cell necrosis, especially in the centriacinar regions (23).

We determined the number of BrdU-positive cells per epithelial basement membrane surface area by using Stereology Toolbox (Morphometrix, Davis, CA) and local vertical sections (2).

Inflammatory and epithelial necrosis indexes. Because we are interested in the relationship between epithelial necrosis and airway inflammation and reparative epithelial cell proliferation, and because epithelial necrosis varied between the groups studied, we calculated a necrosis and inflammation index for all the airways studied, as follows

\[
\text{Index} = (\text{severity score}) \times (\text{extent score})
\]

To examine the relationship between inflammation and necrosis for all airway levels studied, we divided the inflammatory index by the epithelial necrosis index. To examine the relationship between BrdU-positive cells and epithelial necrosis, we divided the numbers of BrdU-positive cells per square millimeter of epithelial membrane in the terminal bronchioles by the terminal broncholar epithelial necrosis index.

Statistical analysis. Unless otherwise stated, we report all data as means ± SE. To check for significant interactions, we analyzed breathing pattern data by using a three-way ANOVA with repeated measures, where treatment (vehicle-treated control, capsaicin-treated) and exposure (FA, ozone) were the grouping factors and time was the repeated measure. Differences between the four groups (vehicle-treated control rats exposed to FA; vehicle-treated control rats exposed to ozone; capsaicin-treated rats exposed to FA; and capsaicin-treated rats exposed to ozone) at each time point were analyzed by using ANOVA and the Student-Newman-Keuls test (SAS, version 6.10; SAS Institute, Cary, NC). We evaluated the semiquantitative scoring system by using the nonparametric Kruskal-Wallis and Mann-Whitney U-tests. We analyzed all other data by ANOVA and Fisher’s least significant difference test (Systat 5 for Macintosh, version 5.2; Systat, Evanston, IL). For all statistical tests, we chose \( P < 0.05 \) as the level of significance.

RESULTS

Effect of capsaicin treatment and ozone inhalation on tracheal SP levels. The effectiveness of our method to deplete SP (and, by implication, C fibers) was verified by quantifying SP concentrations in tracheal homogenates. SP levels were significantly reduced by both capsaicin-treated rats exposed to FA and capsaicin-treated rats exposed to ozone (Fig. 2). The ozone-exposed control group showed a significant decrease of 42%, whereas the ozone-exposed, capsaicin-treated group showed a nonsignificant reduction of 59%. Given the observation that capsaicin-treated rats had a 80% decrease in their baseline airway SP, this would indicate that ozone inhalation in the control rat airway resulted in an ~3.5-fold greater release of SP into the surrounding tissue.

![Tracheal Substance P Levels](image)

Table 2. Baseline values for tidal volume, breathing frequency, and minute ventilation for vehicle-treated control and capsaicin-treated rats before inhaling filtered air or ozone

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>Capsaicin-Treated</th>
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<tbody>
<tr>
<td></td>
<td>FA (n = 10)</td>
<td>O₃ (n = 10)</td>
</tr>
<tr>
<td>VT, ml</td>
<td>1.40 ± 0.12</td>
<td>1.33 ± 0.16</td>
</tr>
<tr>
<td>f, breaths/min</td>
<td>83.1 ± 6.3</td>
<td>84.1 ± 8.9</td>
</tr>
<tr>
<td>VE, ml/min</td>
<td>116.1 ± 8.9</td>
<td>111.4 ± 17.9</td>
</tr>
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</table>

Values are means ± SE, n, no. of rats; FA, filtered air; O₃, ozone; VT, tidal volume; f, breathing frequency; VE, minute ventilation.
remainder of the experimental time. In contrast, f showed a more abrupt return toward baseline and was no longer significantly different from the corresponding FA-exposed control group after 12 h of experimental time (4 h postexposure). There were no significant differences in VT and f responses between the FA-exposed, vehicle-treated control rats; capsaicin-treated, FA-exposed rats; and ozone-exposed, capsaicin-treated rats except in the last 2 h of the experimental period, when the percent change in f for the FA-exposed control group was significantly different from the ozone-exposed capsaicin-treated group. There was also a significant difference in $V_{\dot{E}}$ between the ozone-exposed, capsaicin-treated group and the FA-exposed, capsaicin-treated group at the 16-h experimental time point.

Morphology of nasal cavities and lungs. Overall, both vehicle-treated control rats exposed to ozone and capsaicin-treated rats exposed to ozone showed more severe necrosis of the nasal cavity and more severe inflammation throughout the respiratory tract compared with vehicle-treated control rats exposed to ozone.

There were no significant differences between the vehicle-treated control group and capsaicin-treated group exposed to FA, although they showed occasional mild necrosis and sloughing with minimal intraepithelial infiltration of neutrophils of the nasal transitional epithelium. We attributed these unremarkable epithelial lesions to our head-only inhalation system because its design did not allow us to humidify the air before its entry into the head-only apparatus.

Within the nasal cavity, histological changes ranged from attenuation of the transitional epithelium to sloughing of epithelium into the nasal lumen with associated coagulative necrosis primarily along the nasoturbinates and maxilloturbinates (Figs. 4A, 5A, and 6A). The inflammatory changes within the underlying lamina propria and/or submucosa were characterized by an influx of primarily neutrophils. In addition, a luminal fibrinous exudate admixed with varying

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Fig. 3. Time course of effect of FA and $O_3$ on tidal volume, breathing frequency, and minute ventilation in vehicle-treated controls (A, C, E, respectively) and capsaicin-treated rats (B, D, F, respectively). Values are means ± SE for 9–10 animals. FA-exposed, vehicle-treated (△) and FA-exposed, capsaicin-treated (●) rats were exposed to FA for 16 h. O$_3$-exposed, vehicle-treated (△) and O$_3$-exposed, capsaicin-treated (●) rats were exposed to 1 ppm O$_3$ for 8 h followed by an 8-h FA postexposure period. *Significantly different (P < 0.05) from corresponding FA-exposed control group. Opposite SE bars were omitted for clarity.
amounts of neutrophils was present in the nasal cavity. The luminal exudate tended to be more severe in the capsaicin-treated, ozone-exposed rats compared with the control rats exposed to ozone, but it was not significantly different.

No necrotic cells were observed in bronchi and terminal bronchioles of either FA group. Pathological changes in the bronchi (Figs. 4B, 5B, and 6B) and terminal bronchioles (Figs. 4C, 5C, and 6C) included swelling and vacuolation of bronchial epithelium, with occasional sloughing of necrotic cells into the lumen. There were no differences between the inflammatory scores for the bronchi and terminal bronchioles of the FA groups with only the occasional presence of inflammatory cells within the interstitium of the bronchi and terminal bronchioles. Ozone-induced inflammatory changes included accumulation of predominantly neutrophils, either diffusely distributed or in variably sized aggregates, in and around the bronchi, terminal bronchioles, and adjacent alveolar septa and in perivascular spaces associated with the bronchi.

To examine the relationship between inflammation and necrosis, we examined the calculated ratios of the inflammatory index to the necrosis index for the nasal cavity (Fig. 7A), the bronchi (Fig. 7B), and terminal bronchioles (Fig. 7C). These ratios were not significantly different for the capsaicin-treated and control rats exposed to ozone. There was a nonsignificant trend (P < 0.07) for this ratio to be less in the nasal cavity of capsaicin-treated rats exposed to ozone (Fig. 7A).

Morphometry of lungs. The mean numbers of BrdU-labeled epithelial cells (Fig. 8) for both capsaicin-treated rats and control rats exposed to ozone were significantly greater than the corresponding FA-exposed groups (Fig. 9A). Ozone-exposed, capsaicin-treated rats had 47.26 ± 9.00 BrdU-positive cells/mm² epithelial basement membrane, whereas capsaicin-treated rats exposed to FA had 1.93 ± 0.89 positive cells/mm² basement membrane. Vehicle-treated control rats exposed to ozone were calculated to have 69.03 ± 16.59 BrdU-positive cells/mm² epithelial basement membrane compared with a value of 22.19 ± 6.60 BrdU-labeled cells for control rats exposed to FA. The total number of BrdU-positive cells/mm² epithelial basement membrane for the capsaicin-treated, ozone-exposed group tended to be less than that in the control...
rats exposed to ozone but was not statistically significant.

When expressed as a ratio to the calculated terminal bronchiolar necrosis index, the effect of ozone inhalation on BrdU labeling is abolished and a significant effect of capsaicin treatment is revealed, with the comparison of capsaicin-treated to control being significant for both FA- and ozone-exposed rats (Fig. 9B).

**DISCUSSION**

We utilized neonatal capsaicin treatment in this and our previous study (28) to ablate rats of capsaicin-sensitive sensory C fibers innervating the lung and airways. The tracheal content of SP, a neuropeptide released from capsaicin-sensitive C fibers, was reduced by 80% in the rats treated with capsaicin in the present study, demonstrating the effectiveness of this treatment. However, it should be noted that neonatal capsaicin treatment is known to deplete other peptide markers associated with primary sensory afferents, including neurokinin A, calcitonin gene-related peptide, somatostatin, vasoactive intestinal polypeptide, cholecystokinin, and fluoride-resistant acid phosphatase (26). The profile of peptides released from capsaicin-sensitive C fibers during ozone inhalation will determine, in part, the local physiological effect, with the final response being determined by the balance of the proinflammatory and anti-inflammatory effects of these and other released substances. In addition, neonatal capsaicin treatment is known to ablate primary sensory afferents that innervate not only the lung but other tissues throughout the body and has been reported to diminish the number of small myelinated A-delta fibers located in the dorsal roots of the spinal cord (26). We assume, given the anatomic location of these other fibers, that they play a minor role in the acute response to ozone inhalation; however, this remains undetermined.

In designing this experiment we had to consider several factors to optimize the testing of our overall hypothesis. Previous studies in our laboratory had shown that exposure to 1 ppm ozone, a concentration greater than that found in the ambient environment, for 8 h was necessary to give us sufficient injury to attain measurable values for some of the end points studied (23). A postexposure period of 8 h was chosen because of our desire to examine BrdU labeling while...
also examining injury and inflammation and was based on earlier data from two inhalation studies of chamber-exposed rats. The first was our previous study of capsaicin treatment on ozone-induced injury and inflammation (30) that demonstrated capsaicin-induced differences at 4 h, but not at 16 h, after an 8-h exposure to 1 ppm ozone. In the second study (unpublished observations), maximal BrdU labeling of terminal bronchiolar epithelial cells was found to occur between 4 and 16 h after the end of an 8-h ozone exposure period.

We utilized an anesthetized rat preparation in this study to optimize the accurate continuous measurement of breathing pattern over the entire period of the experiments (16 h) without the confounding factors associated with the prolonged restraint of conscious animals. However, the use of anesthesia in this experiment resulted in a depressed $V_E$ compared with that in conscious restrained rats (19, 32) and may have contributed to the observation of milder centriacinar lesions in this study compared with the more severe lesions we previously reported in chamber-exposed, conscious rats at the same exposure concentration and period (30). In addition, the use of bronchoalveolar lavage before lung fixation in our previous study (30) may have also contributed to the more severe lesions reported in that study. As a result, we did not use lung lavage in the present study and used a modified vascular fixation technique that delivers fixative to the pulmonary and airway vasculature and minimizes the displacement of airway cells while optimizing the preservation of airway structure (3).

Our data show that capsaicin-sensitive C fibers are necessary for ozone-induced reflex rapid, shallow breathing in Wistar rats. Ozone inhalation in capsaicin-treated rats had no effect on $V_T$ and $f$ compared with in the corresponding FA control group. In contrast, ozone inhalation in the control rats resulted in a progressive decrease in $V_T$ and an increase in $f$, with maximal percent change occurring at 8 h of exposure. At 8 h of exposure, $f$ in the ozone-exposed group had increased from the preexposure value of 82 breaths/min to 106 breaths/min (an increase of 23%). This percent increase in $f$ is similar to the 21% increase in $f$ previously reported in awake, restrained rats exposed to ozone (19). In that study, $f$ showed an increase after only 0.8 ppm ozone for 3 h, but the actual $f$ values increased.
from 132 breaths/min in control animals to 167 breaths/min in exposed animals. We attribute the lower baseline and peak-exposure \( f \) values observed in this study to an anesthesia-induced decrease in \( f \). Interestingly, in our control rats exposed to ozone, we did not observe a ozone-induced drop in \( V_E \) that has been reported by other investigators (19) and appears to be related to an ozone-induced drop in metabolism (19). We believe that the lack of an effect on \( V_E \) in the present study is related to our use of anesthesia. One possibility is that anesthesia may have lowered metabolism to the extent that a further ozone-induced reduction was not possible. Alternatively, the observed reduction in \( V_E \) and metabolism in conscious rats may be the result of a stress-induced reduction in metabolism that is dependent on the perception of discomfort that is eliminated by anesthesia.

Tepper et al. (31) observed an exacerbation of ozone-induced rapid, shallow breathing and an attenuation of ozone-induced airway hyperresponsiveness and airway permeability in capsaicin-treated guinea pigs. In this
study, Tepper et al. treated adult guinea pigs with progressively increasing doses of capsaicin and 10 days later exposed them to 1.0 or 2.0 ppm ozone for 2 h. Although the attenuation of ozone-induced airway hyperresponsiveness and airway permeability is consistent with neuropeptides playing a role in ozone-induced airway constrictor and permeability responses in guinea pigs, the exacerbation of ozone-induced reflex-induced rapid, shallow breathing may be due to species differences and/or differences in the effects of adult vs. neonatal capsaicin treatment. Neonatal capsaicin treatment is known to ablate small nonmyelinated sensory neurons in the spinal cord and vagus nerves (18, 26). In contrast, adult capsaicin treatment acts by stimulating a massive secretion of neuropeptides, interfering with axonal transport of neuropeptides and blocking action potential conduction by hyperpolarizing the axonal membrane (26). The rate at which the nerve recovers from each of these effects is not known. If the capacity of the axon to transmit action potentials recovered more rapidly than the depletion of neuropeptides, then reflex-induced rapid, shallow breathing would be present and may be exacerbated if, as in our rats, the lack of neuropeptides resulted in more severe airway injury.

Studies from our laboratory (7, 27) have demonstrated that ozone-induced rapid, shallow breathing in dogs is mediated by vagal C fibers innervating the conducting airways. On the basis of a published theoretical model of regional uptake of ozone (21), we expected that the rapid, shallow breathing pattern during ozone inhalation would reduce the total respiratory tract uptake of ozone by reducing the uptake in the distal airways while leaving the uptake of the conducting airways relatively unchanged. On the basis of this theoretical prediction, if rapid, shallow breathing was the only C-fiber-evoked protective response, then the ozone-induced lesion in the centriacinar region would be greater in the capsaicin-treated rats, whereas the severity of lesions in the conducting airways would be similar in capsaicin-treat and control rats. Consistent with this prediction was our observation of more severe centriacinar lesions in capsaicin-treated rats in our previous study (30) and a trend toward more severe centriacinar lesions in capsaicin-treated rats in the present study. However, we also observed more severe ozone-induced lesions in the transitional epithelium of the nasal cavity in our present study and in the ciliated epithelium of the bronchi in our previous study (30). These observations suggest that, although C-fiber-mediated rapid, shallow breathing acts to limit the deep lung penetration of ozone, other C-fiber-mediated responses may also act simultaneously to protect the conducting airways.

One possible C-fiber-mediated mechanism that would act to protect the conducting airways may be related to C-fiber control of airway mucous secretions. The ablation of lung C fibers would be expected to abolish C-fiber-induced reflex (5) and neuropeptide-induced (1) increases in airway mucous secretion. Mucous may play a protective role by providing a layer of continually renewable substrate that scavenges inhaled pollutants such as ozone (10). Ozone is known to react with multiple components within the mucous layer, including polyunsaturated fatty acids contained in lipids and numerous amino acids found in proteins (24). However, this mechanism would be expected to protect only the large conducting airways where mucous secreting cells are located and would not be expected to significantly...
alter delivery of ozone to terminal bronchioles and alveolar ducts (20).

Another possible C-fiber-mediated protective response may be through the C-fiber’s role in regulating airway blood flow. The ablation of lung C fibers would be expected to abolish C-fiber-induced reflex and neuropeptide-induced increases in airway blood flow (1, 6) if present. Ozone inhalation has been shown to increase bronchial blood flow in sheep (28) and induce airway erythema in human subjects (29). Whether ozone inhalation induces an increase in bronchial blood flow in rats and whether such an increase in bronchial blood flow is modulated by neuropeptides is unknown. Evans et al. (9) have shown that ozone does not increase tracheal vascular permeability in rats; whether this means that ozone inhalation does not increase airway blood flow is unclear. An increase in blood flow by itself would play a protective role by increasing the delivery of small, highly permeable soluble antioxidants, such as uric and ascorbic acid, to the airway mucosa.

Our experimental design allowed us to evaluate the effect of ozone inhalation on SP levels in the airways. We observed a reduction in SP levels in both ozone-exposed groups compared with their corresponding FA-exposed group, with a 3.5 times greater reduction in control rats compared with capsaicin-treated rats. The observed decrease in SP levels with ozone exposure is most likely the result of prolonged excitation of lung C fibers and the gradual release of neuropeptides into airway tissues and the subsequent cleavage of SP by neutral endopeptidases (1). The release of neuropeptides from C fibers has been shown to increase airway mucosal blood flow and permeability (1) and to augment neutrophil chemotaxis and function (22). These proinflammatory effects would be expected to amplify the inflammatory response at sites of neuropeptide release. On initial inspection, the data from this study and our previously published study (30) do not support a proinflammatory role for sensory C fibers in ozone-induced injury. In these two studies, elimination of C fibers and associated neuropeptides actually resulted in an increase and not a decrease in ozone-induced inflammation. We believe this apparent contradiction is the result of the attenuation of respiratory-tract-protective mechanisms in the capsaicin-treated rats. This reduction in C-fiber-dependent protective mechanisms would result in greater epithelial injury in the capsaicin-treated rats exposed to ozone. The more severe injury to the airway epithelial cells would cause an increased release of the neutrophil chemoattractants, especially chemokine-induced neutrophil chemoattractant (8). Thus the increased release of chemokine-induced neutrophil chemoattractant may overwhelm any decreased proinflammatory effect from a reduction in C fibers after neonatal capsaicin treatment, resulting in the more severe inflammatory response that we observed. This speculation is supported by our post hoc analysis of epithelial inflammation in relation to epithelial necrosis (Fig. 6). The results of this analysis suggest that, for a given degree of necrosis, the degree of inflammation was no greater when capsaicin-treated and control rats exposed to ozone are compared, with a trend toward less inflammation in the nasal cavity in relation to the degree of necrosis (Fig. 7). These findings indicate that sensory C-fiber innervation of the bronchi and terminal bronchioles does not play an important role in the ozone-induced inflammation present at 8 h after exposure but may play some proinflammatory role in the nasal cavity.

Given the greater degree of inflammation and the trend toward greater necrosis within the terminal bronchioles in capsaicin-treated rats, we would anticipate more BrdU-labeled cells than in control animals (25). Surprisingly, the total number of BrdU-labeled cells in the terminal bronchioles for the capsaicin-treated rats exposed to ozone was not significantly different compared with that in the control group exposed to ozone (Fig. 9A) and, when expressed in proportion to the degree of necrosis, was significantly less (Fig. 9B). Interestingly, capsaicin treatment by itself appeared to significantly affect the basal turnover of epithelial cells within the terminal bronchioles (Fig. 9, A and B). These observations suggest that the tonic release of neuropeptides from sensory C fibers in the terminal bronchioles plays an important role in modulating normal epithelial cell turnover and the ability of the epithelium to proliferate in response to ozone-induced injury.

Recently, Kim et al. (16) have shown that the treatment of adult guinea pigs with capsaicin retards the proliferation and repair of tracheal epithelium after mechanical injury. Consistent with the capsaicin-induced effect on epithelial repair, Kim et al. (17) and White et al. (33) have shown that SP, neurokinin A, and calcitonin gene-related peptide modulate the migration and proliferation of guinea pig and human airway epithelial cells in vitro. Therefore, a reduced tonic release of neuropeptides in capsaicin-treated animals may actually slow epithelial repair after ozone injury through decreased mitogenesis and lead to a more prolonged and severe inflammatory response.

In summary, our data support that capsaicin-sensitive lung C fibers initiate the reflex of rapid, shallow breathing and release neuropeptides after inhalation of an irritant such as ozone. Our data suggest that this ozone-induced release of neuropeptides does not significantly contribute to ozone-induced airway inflammation. In addition, our data suggest that the tonic release of neuropeptides modulates normal epithelial turnover and thereby contributes to terminal bronchiolar epithelial repair after ozone inhalation.

We thank Brian Tarkington and Tim Duvall of the California Primate Research Center, Air Pollutant Exposure Facility, for expert technical assistance in the development of the ozone-exposure system used in these studies. We thank Dr. Anja Sterner-Kock for her expertise and time taken to analyze the tracheal substance P levels. We also thank the laboratory interns who made the 22-h-long experiments possible. We especially thank Jennifer Loomis, Mark Lutz, Jenny Lin, Collette Brown, Tan Phan, and Erin Colson for their dedication and willingness to work, often after normal duty hours. Finally, we thank William Walby for proofreading the manuscript and acting as a sounding board for the ideas it expresses.

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