Role of tachykinins in airway responses to ozone in rats

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J. Appl. Physiol. 85(2): 442–450, 1998.—Previous studies that used neonatal capsaicin (Cap) treatment to ablate C fibers indicate that C fibers act to inhibit lung damage and airway hyperresponsiveness after ozone (O3) exposure in rats. The purpose of this study was to determine 1) the role of tachykinins in these protective effects and 2) whether differences in minute ventilation (Ve) during O3 exposure might account for the effect of Cap. In the first study, male Sprague-Dawley rats were exposed to 1 part/million O3 or air for 3 h. Four hours later, a bronchoalveolar lavage (BAL) was performed or airway responsiveness was measured. Rats were treated with CP-99994 and SR-48968, selective neurokinin-1- and -2-receptor antagonists, respectively, or with vehicle (Veh). O3 caused an increase in the number of neutrophils recovered from BAL fluid in both the Veh-treated and tachykinin-receptor antagonist (TKRA)-treated rats, but the number of neutrophils was approximately twofold greater in the TKRA-treated rats. In contrast, TKRA treatment had no effect on baseline pulmonary mechanics or airway responsiveness. After O3 exposure, the number of neutrophils in BAL fluid was also greater in Cap- than in Veh-treated rats. O3 reduced Ve in both Veh- and Cap-treated rats, but the response was greater (reduction of 44.7 ± 3.7 vs. 27.8 ± 6.8%) and occurred earlier (10 vs. 70 min) in Cap- than in Veh-treated rats (P < 0.02). These results suggest that tachykinins mediate protective effects of C fibers against O3-induced lung inflammation. The results also indicate that the more pronounced effect of O3 on BAL neutrophils in Cap-treated rats is not the result of a greater inhaled dose of O3 resulting from greater Ve.

C fibers, a class of unmyelinated sensory neurons, innervate the lungs and airways of many species, including humans. The terminal ramifications of these neurons are found underneath and between epithelial cells, around blood vessels, and near airway smooth muscle and mucus glands (2, 24). In response to stimulation, the tachykinins, substance P (SP) and neurokinin A (NKA), are released from the peripheral endings of these neurons. The biological effects of these peptides in the lung and airways are diverse. Tachykinins can induce bronchoconstriction, increase microcirculation, cause mucus secretion, and increase vascular permeability (19). Stimulation of C fibers also causes apnea, followed by rapid, shallow breathing (33). C fibers are stimulated by various inhaled irritants including ozone (O3) (4) as well as by many endogenous inflammatory mediators (19). In addition, SP is released into the airway lavage fluid of humans exposed to O3 (12).

Neonatal capsaicin (Cap) pretreatment causes degeneration of C fibers and decreases neuropeptide content in the lungs and airways (24, 27). Our laboratory (15) has previously reported that, in Sprague-Dawley rats treated neonatally with Cap, airway hyperresponsiveness to inhaled methacholine is induced by exposure to concentrations of O3, which do not affect responsiveness in control rats. Neonatal Cap treatment also results in more airway epithelial injury and more inflammation after O3 exposure, as determined by pathology and bronchoalveolar lavage (BAL) in Wistar rats (37). Taken together, these results suggest that C fibers protect the airways during exposure to O3, minimizing airway injury and inflammation and inhibiting the development of airway hyperresponsiveness. C fibers have also been shown to play a protective role in the responses to other inhaled irritants such as sulfur dioxide (22, 23), hydrogen sulfide (30), and acrolein (41).

One aim of this study was to determine whether tachykinins were important in mediating these protective effects of C fibers against O3-induced airway inflammation and airway hyperresponsiveness in rats. To this end, we treated rats with tachykinin-receptor antagonists (TKRAs) or the vehicle (Veh) used to dissolve them, exposed them to O3, or air, and then either performed a BAL or instrumented them for the measurement of pulmonary mechanics and responsiveness to inhaled aerosolized methacholine. The TKRAs, CP-99994 and SR-48968, which possess high potency and selectivity in blocking the neurokinin-1 (NK1) and -2 (NK2) receptors, respectively (8, 26), were employed. Because our experiments were performed in Sprague-Dawley rats, whereas those of Sterner-Kock et al. (37) were performed in Wistar rats, and because differences in responses to tachykinins and to O3 have been observed among strains of rat (9, 16, 39, 42), we also examined the effect of neonatal Cap treatment on O3-induced alterations in BAL cells and protein and confirmed the results of Sterner-Kock et al. (37) indicating that ablation of C fibers causes more lung injury and inflammation after O3 inhalation.

One explanation for the greater inflammatory response of the Cap-treated rats is that a larger dose of O3 is delivered to the lungs of these rats despite a similar O3 concentration. The dose of O3 delivered to the lungs is a function of both O3 concentration and minute ventilation (Ve) (47). During O3 exposure, rats decrease their Ve (1, 25). Because O3 stimulates C fibers and stimulation of C fibers alters Ve (4, 33), it is possible that C fibers are the sensory arm of O3-induced changes...
in \( \dot{V} \text{e} \). If so, one might expect \( \dot{V} \text{e} \) to be reduced during O₃ exposure in normal rats, but not in Cap-treated rats lacking C fibers, leading to an overall greater dose of O₃ in the Cap-treated rats. To test this hypothesis, we measured \( \dot{V} \text{e} \) and its components, tidal volume (\( V_T \)) and breathing frequency (\( f \)), during exposure of awake Veh- and Cap-treated rats to O₃ or air.

**METHODS**

Animals. This study protocol was approved by the Harvard Medical Area Standing Committee on Animals. For the studies with TKRAs, pathogen-free Sprague-Dawley rats were obtained from Charles River Breeding Laboratories (Wilmington, MA) and were housed in group cages. For the study with neonatal Cap treatment, pregnant female Sprague-Dawley rats were purchased from Charles River Laboratories (Belmont, CA). The lower limit of detection of SP was performed by using methods described by Folkesson and assay for SP content by an enzyme-linked immunosorbent assay. SP was extracted from the lungs as previously described (18). The enzyme-linked immunosorbent assay for SP was performed by using methods described by Folkesson et al. (10). The antibody for SP was purchased from Peninsula Laboratories (Belmont, CA). The lower limit of detection of this assay was 3 fmol of SP, and the assay has <0.01% cross-reactivity with NKA (18). Cap pretreatment significantly reduced the SP content of the lungs from 2.83 ± 0.5 to 1.75 ± 0.3 pmol/g tissue (P < 0.001).

O₃ exposure. Exposure to O₃ or filtered room air was conducted in a stainless steel chamber with a Plexiglas door on the front (−145 liters in volume). O₃ was generated by passing dry 100% O₂ through ultraviolet light and mixing it with filtered room air in the chamber. Chamber atmosphere was drawn continuously via a sampling port, and O₃ concentration was measured by an O₃ chemiluminescent analyzer (model 49, Thermo Electron Instruments, Hopkinton, MA), which was calibrated by an ultraviolet photometric O₃ calibrator (model 49PS, Thermo Electron Instruments).

Experimental protocol. Two different studies were performed. The purpose of the first study (TKRA) was to determine whether tachykinins act to limit airway injury and inflammation induced by O₃ and thereby limit the degree of airway hyperresponsiveness induced by O₃. In this study, rats were treated with CP-99994 (0.75 mg/kg ip) and SR-48968 (0.75 mg/kg ip) or with Veh 15 min before exposure to filtered air or O₃. The rats were then exposed to air or O₃ [1 part/million (ppm), for 3 h] and were retreated with the same doses of CP-99994 and SR-48968 or the Veh immediately after the exposure to ensure continued receptor blockade. These doses of TKRAs were chosen based on reports that indicated their effectiveness in inhibiting responses to SP and NKA in rats and guinea pigs (8, 13, 26, 36). Four hours after the air or O₃ exposure had ended, rats were killed, and BAL was performed. Alternatively, 2 h after exposure, rats were anesthetized for the measurement of airway responsiveness to inhaled methacholine, followed by administration of intravenous SP to confirm the efficacy of the antagonists. The time required to anesthetize the animals, perform the necessary surgery, instrument them, and perform the measurements of responsiveness was such that the measurements of the steep part of the dose-response curve coincided in time with BAL performed in the other animals.

The purpose of the second study was to confirm, in Sprague-Dawley rats, reports about Wistar rats indicating that Cap-treated rats that lack C fibers have increased airway injury and airway inflammation after O₃ exposure (37). We also sought to determine whether differences in \( \dot{V} \text{e} \) during O₃ exposure might contribute to these enhanced responses. Two cohorts of rats were used in this study (Cap). In the first cohort, adult rats with or without neonatal Cap treatment were exposed either to 1 ppm O₃ or to filtered air for 3 h. To evaluate inflammatory changes induced by O₃, BAL was performed 4 h after the end of O₃ exposure. In the second cohort, adult rats treated neonatally with either Cap or Veh were placed in head-out restraining tubes that served as body plethysmographs. Once the animal was in the tube, the cranial end of the tube was inserted into a port in the door of the O₃-exposure chamber, so that animals breathed air from within the chamber. We then monitored \( \dot{V} \text{e} \) for 45 min before O₃ was introduced into the chamber. O₃ exposures (2 ppm) were for 90 min followed by a 45- to 90-min recovery period during which we continued to monitor \( \dot{V} \text{e} \). For these experiments, the washin of O₃ was such that 2 ppm was reached within 5 min of initiating the exposure. The washout was such that O₃ had decreased to <0.02 ppm within 5 min after the O₃ flow to the chamber was stopped. Other Cap- and Veh-treated rats were treated identically but were exposed to filtered air only.

Measurements of pulmonary mechanics and airway responsiveness to inhaled aerosolized methacholine were performed as previously described (15). Briefly, rats were anesthetized with pentobarbital sodium (64.8 mg/kg ip), tracheostomized with a tubing adapter (14 gauge, 1.65-mm ID), and mechanically ventilated with a rodent ventilator (Harvard Apparatus, Natick, MA) at 8 ml/kg and 60 breaths/min. End-expiratory pressure was maintained at 3.5 cmH₂O throughout the experiment. The left carotid and right jugular vein were catheterized for blood pressure measurement and drug administration, respectively. The chest wall was opened widely to expose the lungs to atmospheric pressure, and the rat was then placed in a body plethysmograph. Plethysmograph pressure relative to a reference bottle of a similar size was measured with a differential pressure transducer (model LCVR, Cefescho, Canoga Park, CA) and used as a volume signal. Flow was calculated by electrical differentiation of the volume signal. Transpulmonary pressure was obtained with a second pressure transducer (Cefescho) placed between a side tap in a tracheal cannula and the inside of the plethysmograph. All three signals were recorded on a strip chart (model R56SP, General Scanning, Watertown, MA) and fed to a computer for calculation of dynamic compliance (Cdyn) and pulmonary conductance (GL), according to the method of Van Neergard and Wirz (44, 45).

Airway responsiveness was measured as follows. A breath equal to three times \( \text{Vt} \) was given to provide a constant volume history. Baseline measurements of GL and Cdyn were obtained 1 min later. Animals were then given 20 tidal breaths of aerosolized saline generated in an acorn nebulizer. A flow rate of 4.5 l/min was used to generate the aerosol. GL
and Cdyn were then measured repeatedly over the next 3 min, and the peak response was recorded. The procedure was then repeated by using methacholine solutions in the nebulizer, beginning with 0.1 mg/ml and increasing in one-half log units up to 300 mg/ml. The time interval between doses was sufficient for GL to return to within 10% of its original value or the time interval was 10 min, whichever came first. GL and Cdyn values measured before the methacholine challenge are reported as baseline values. The methacholine doses required to produce a 50% decrease of GL and Cdyn were determined by log-linear interpolation between the two doses bounding the point at which GL or Cdyn was exactly 50% of its baseline value.

At the end of the methacholine challenge, changes in blood pressure caused by intravenous infusions of increasing concentrations of SP were measured to confirm the efficacy of the TKRAs. The doses of SP ranged from 10^{-12} to 10^{-10} mol/kg. SP was injected over 20 s, and each administration was separated by 5 min. This time interval was sufficient for blood pressure to return to the baseline level.

BAL. Animals were given a lethal dose of pentobarbital sodium (64.8 mg in 1.0 ml) intravenously. The chest wall was opened, and the lungs were then lavaged twice with 5 ml of saline. For the lavages, saline was injected and withdrawn gently over 30 s. The recovered BAL fluid was centrifuged at 1,200 rpm at 4°C for 10 min. Cell pellets were resuspended in 5 ml of saline, and the total number of cells was counted by a hemocytometer. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 5 min (Cytospin2, Shandon, Sewickley, PA), air-dried, and stained with Wright-Giemsa. Cells were monitored under 800× and 1,200× magnification. Aliquots of cells were also centrifuged onto glass slides at 800 rpm for 5 min (Cytospin2, Shandon, Sewickley, PA), air-dried, and stained with Wright-Giemsa (LeukoStat, Fisher Scientific, Pittsburgh, PA). Cell differentiation was determined by the counting of 1,200 cells under ×400 magnification. Total protein concentration in the BAL supernatant was determined by the Bradford technique.

Monitoring of V˙E. Rats were placed in a Plexiglas restraining tube, which served as a head-out flow plethysmograph. The tube was fitted with a silicone rubber gasket designed to fit snugly around the animal’s neck and seal the head from the rest of the body. Once the animal was in the tube, a large piston was moved into placed behind the animal. The piston served to prevent the animal from moving and to seal the body chamber from the outside air. Air, displaced at the body surface as the animal breathed, passed across a pneumotachograph (Climacor, Harvard, MA) connected to the mouthpiece and to a pneumotachograph (Buxco, Troy, NY) that computed V˙E, Vt, f, and inspiratory (T) and expiratory time (T) on a breath-by-breath basis and reported the average of each of these values every minute.

Chemicals. Methacholine chloride and SP were purchased from Sigma Chemical (St. Louis, MO) and were dissolved in saline. CP-99994 and SR-48968 were synthesized at Merck Frost and were dissolved in a 1:1 solution of saline and polyethylene glycol (average mol wt 200; Sigma Chemical). Cap was obtained from Spectrum Chemical Manufacturing.

Statistical analysis. Results are expressed as means ± SE. For baseline values of pulmonary mechanics and log 50% effective dose data, analysis of variance was employed to detect differences across all groups in the TKRA study. The effect of TKRAs on changes in blood pressure induced by SP was examined by using Student’s t-test. For BAL data, analysis of variance was first employed to detect differences across all groups. If a statistically significant result was observed, follow-up t-tests were used to determine the significance of differences across groups. The Bonferroni correction was employed to correct for multiple comparisons. Because there were no significant differences in any BAL parameter between the two air-exposed groups, the air-exposed animals were combined for these comparisons. For ventilatory parameters, mean values over the 20 min immediately preceding O3 exposure were determined for each animal, and data are reported as percent changes from those values. In each animal, 5-min averages around the 10-, 30-, 50-, 70-, and 90-min time points for O3 exposure were computed. The effect of Cap treatment on these parameters was then assessed by repeated-measures analysis of variance. A P value of <0.05 was considered statistically significant.

RESULTS

TKRA study. To determine whether tachykinins released from C fibers act to limit O3-induced airway injury and inflammation, we examined the effect of TKRAs on BAL cells and protein in O3-exposed animals. The profile of cells recovered from BAL was affected by both O3 exposure and TKRA treatment (Table 1). Analysis of variance indicated significant differences across the groups in the number of BAL neutrophils and ciliated epithelial cells and the BAL protein concentration, whereas total cell numbers, lymphocytes, and macrophages were unchanged. Treatment with TKRAs did not affect either the BAL cell profile or the BAL protein concentration in air-exposed rats. O3 exposure increased the number of neutrophils in both the Veh- and the TKRA-treated rats, compared with air-exposed controls. However, the number of neutrophils recovered from the TKRA-treated rats exposed to O3 was significantly greater than that from the Veh-treated rats exposed to O3. O3 exposure also increased BAL epithelial cells and BAL protein concentration in both the Veh- and the TKRA-treated rats, compared with air-exposed controls (Table 1). However, in contrast to the effect of TKRA on BAL neutrophils,

Table 1. Effects of O3 and inhibition of tachykinin receptors on no. of cells and total protein concentration in bronchoalveolar lavage fluid

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Treatment</th>
<th>Body Wt. g</th>
<th>Total Cells</th>
<th>Macrophages</th>
<th>Neutrophils*</th>
<th>Lymphocytes</th>
<th>Epithelial Cells*</th>
<th>Total Protein, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>Vehicle</td>
<td>301 ± 3</td>
<td>12.8 ± 0.42</td>
<td>12.3 ± 0.48</td>
<td>0.07 ± 0.02</td>
<td>0.22 ± 0.04</td>
<td>0.06 ± 0.01</td>
<td>0.31 ± 0.02</td>
</tr>
<tr>
<td>Air</td>
<td>TKRA</td>
<td>302 ± 3</td>
<td>11.4 ± 0.55</td>
<td>11.0 ± 0.54</td>
<td>0.07 ± 0.01</td>
<td>0.18 ± 0.02</td>
<td>0.05 ± 0.01</td>
<td>0.34 ± 0.04</td>
</tr>
<tr>
<td>O3</td>
<td>Vehicle</td>
<td>301 ± 3</td>
<td>12.0 ± 0.64</td>
<td>11.0 ± 0.67</td>
<td>0.55 ± 0.07†</td>
<td>0.24 ± 0.03</td>
<td>0.17 ± 0.03†</td>
<td>0.51 ± 0.03†</td>
</tr>
<tr>
<td>O3</td>
<td>TKRA</td>
<td>303 ± 4</td>
<td>14.0 ± 1.07</td>
<td>12.5 ± 0.94</td>
<td>1.07 ± 0.13‡</td>
<td>0.23 ± 0.04</td>
<td>0.27 ± 0.05‡</td>
<td>0.51 ± 0.03‡</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 6 animals. Body weight of animal on day of experiment is shown. TKRA, tachykinin-receptor antagonist. Cells are expressed as no. of cells in bronchoalveolar lavage fluid × 10^6. *P < 0.001 by ANOVA; †P < 0.02, ozone (O3)-exposed, vehicle-treated group compared with air-exposed groups (vehicle and TKRA combined); ‡P < 0.02, O3-exposed, TKRA-treated group compared with air-exposed groups; §P < 0.02 compared with O3/vehicle.
Table 2. Effects of O₃ and inhibition of tachykinin receptors on airway responsiveness to inhaled methacholine

<table>
<thead>
<tr>
<th>Exposure</th>
<th>Treatment</th>
<th>Body Wt, g</th>
<th>Baseline Gₛ, cmH₂O·ml⁻¹·s</th>
<th>log ED₅₀Gₛ, mg/ml</th>
<th>Baseline Cdyn, ml/cmH₂O</th>
<th>log ED₅₀Cdyn, mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>Air</td>
<td>Vehicle</td>
<td>317 ± 8</td>
<td>9.69 ± 0.22</td>
<td>0.64 ± 0.20</td>
<td>0.546 ± 0.027</td>
<td>0.80 ± 0.11</td>
</tr>
<tr>
<td>Air</td>
<td>TKRA</td>
<td>327 ± 7</td>
<td>9.85 ± 0.12</td>
<td>0.76 ± 0.15</td>
<td>0.542 ± 0.020</td>
<td>0.72 ± 0.17</td>
</tr>
<tr>
<td>O₃</td>
<td>Vehicle</td>
<td>314 ± 9</td>
<td>9.51 ± 0.30</td>
<td>0.66 ± 0.05</td>
<td>0.581 ± 0.028</td>
<td>0.67 ± 0.03</td>
</tr>
<tr>
<td>O₃</td>
<td>TKRA</td>
<td>327 ± 8</td>
<td>9.72 ± 0.32</td>
<td>0.71 ± 0.08</td>
<td>0.593 ± 0.013</td>
<td>0.74 ± 0.07</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 4 animals for air-exposed and 6 for O₃-exposed groups. Gₛ, pulmonary conductance; Cdyn, dynamic compliance; ED₅₀Gₛ, and ED₅₀Cdyn, inhaled methacholine dose required to decrease Gₛ and Cdyn, respectively, by 50% of their baseline values. No statistically significant difference was detected.

Table 3. Effects of intravenous substance P infusion on mean arterial blood pressure (mmHg)

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Baseline Value</th>
<th>Substance P 10⁻¹² mol/kg</th>
<th>Substance P 10⁻¹¹ mol/kg</th>
<th>Substance P 10⁻¹⁰ mol/kg</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vehicle</td>
<td>112 ± 2</td>
<td>110 ± 3</td>
<td>93 ± 3</td>
<td>78 ± 3</td>
</tr>
<tr>
<td>TKRA</td>
<td>118 ± 3</td>
<td>118 ± 3</td>
<td>118 ± 3*</td>
<td>86 ± 3*</td>
</tr>
</tbody>
</table>

Values are means ± SE; n = 10 animals. *P < 0.05 compared with vehicle group.

To determine whether the effective dose of O₃ delivered to the lungs might be different in Cap- and Veh-treated rats, we measured Vₑ and examined the pattern of breathing during air or O₃ exposure. Table 5 shows mean baseline ventilatory parameters measured during the 20 min immediately before O₃ (or air) exposure (after the animals were allowed 25 min to settle in the restraining tubes). No significant differences between Cap- and Veh-treated rats were observed.

In Cap- and Veh-treated rats exposed to filtered air, there was substantial minute-to-minute variability in Vₑ, but no significant trend over time in either group and no significant effect of Cap treatment (Fig. 1). Similarly, no significant changes in Vₑ or Tₑ were observed with time in air-exposed animals (data not shown).

Table 3. Effects of intravenous substance P infusion on mean arterial blood pressure (mmHg)

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after O₃ exposure but not at any other time point (Fig. 2). Overall, there was no significant effect of Cap treatment on f. Even though f was not altered by O₃ exposure, the timing of the breath was altered. In particular, Tᵢ increased in the first 10 min of O₃ exposure and then decreased to values well below baseline over the next 60 min (Fig. 2). In contrast, Tₑ increased over the course of O₃ exposure (Fig. 2), although the changes were much more variable among animals. There were no statistically significant differences between Cap- and Veh-treated animals in the changes in Tᵢ and Tₑ induced by O₃ exposure.

**DISCUSSION**

The results of the present study demonstrate that combined inhibition of NK₁ and NK₂ tachykinin receptors during exposure to O₃ augments neutrophil influx into the lungs and airways (Table 1). In contrast, combined inhibition of NK₁ and NK₂ receptors does not result in the development of airway hyperresponsiveness after O₃ exposure (Table 2). Neonatal Cap pretreatment also resulted in greater O₃-induced neutrophil influx and protein extravasation (Table 4). In addition, rats treated neonatally with Cap had more pronounced decreases in Vₑ during O₃ exposure than did control rats (Fig. 2).

Sterner-Kock et al. (37) have previously reported that, in Wistar rats treated neonatally with Cap, 1 ppm O₃ exposure for 8 h caused more severe interstitial inflammation and epithelial necrosis, more protein in BAL fluid, and increased neutrophils in BAL fluid compared with Veh-treated rats. We also observed more neutrophils and more protein in BAL fluid in Cap-treated Sprague-Dawley rats (Table 4) exposed to 1 ppm O₃ for only 3 h. These observations with Cap-treated animals suggest that, in two different strains of rat, C fibers protect the lungs against O₃-induced injury and consequent inflammation. The observation that, after O₃ exposure, treatment with TKRAs also increases the recovery of neutrophils in BAL fluid (Table 1) suggests that tachykinins mediate at least part of this protective effect provided by C fibers.

We had hypothesized that one way in which C fibers might be mediating these protective effects was through changes in Vₑ. In particular, we reasoned that stimulation of C fibers by O₃ might lead to a decrease in Vₑ in normal rats, which would not be present in the Cap-treated rats. The net effect would be that the Cap-treated rats received a greater dose of O₃, resulting in greater inflammatory responses. Our results do not support this hypothesis. After a delay of ~70 min, Vₑ decreased to ~70% of values measured in the 20-min period immediately before O₃ exposure in Veh-treated rats (Fig. 2). However, Vₑ decreased earlier and to a greater extent in the Cap-treated rats (Fig. 2). Tepper et al. (38) reported similar results in Cap-treated guinea pigs exposed to O₃. Thus the increased inflammatory response to O₃ in Cap-treated rats (Table 4) is not the result of a greater inhaled dose of O₃. In contrast, increased inflammation develops despite a lower inhaled dose.

The observations that Vₑ and Vₜ decreased to a greater extent in Cap- than in Veh-treated rats and that changes in the timing of the breath were not altered by Cap treatment indicate that C fibers are not the sensory arm of the changes in Vₑ or ventilatory timing evoked by O₃ in rats. What is responsible for these changes? Other receptors, for example, rapidly adapting pulmonary stretch receptors and upper airway receptors, are also stimulated during O₃ exposure (1, 4). In dogs, data obtained during CO₂ rebreathing after O₃ exposure suggest that slowly adapting pulmonary stretch receptors are sensitized by O₃ (31), although direct recordings from slowly adapting pulmonary stretch receptors do not indicate any consistent activation of these fibers by O₃ (4). The effect of vagotomy or vagal cooling on ventilatory responses to O₃ exposure is illustrated in Figure 2. Overall, there was no significant effect of Cap treatment on f. Even though f was not altered by O₃ exposure, the timing of the breath was altered. In particular, Tᵢ increased in the first 10 min of O₃ exposure and then decreased to values well below baseline over the next 60 min (Fig. 2). In contrast, Tₑ increased over the course of O₃ exposure (Fig. 2), consistent with other reports (1, 25).

However, Vₑ decreased earlier and to a greater extent in the Cap-treated rats (Fig. 2). Tepper et al. (38) reported similar results in Cap-treated guinea pigs exposed to O₃. Thus the increased inflammatory response to O₃ in Cap-treated rats (Table 4) is not the result of a greater inhaled dose of O₃. In contrast, increased inflammation develops despite a lower inhaled dose.

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**Table 5.** Effect of capsaicin on baseline ventilatory parameters

<table>
<thead>
<tr>
<th></th>
<th>Vₑ, ml/min</th>
<th>Vₜ, ml</th>
<th>f, breaths/min</th>
<th>Tᵢ, s</th>
<th>Tₑ, s</th>
<th>Weight, g</th>
</tr>
</thead>
<tbody>
<tr>
<td>Veh</td>
<td>205.5 ± 17.3</td>
<td>1.58 ± 0.13</td>
<td>135.8 ± 9.1</td>
<td>0.257 ± 0.026</td>
<td>0.264 ± 0.024</td>
<td>328.1 ± 7.1</td>
</tr>
<tr>
<td>Capsain</td>
<td>201.1 ± 17.6</td>
<td>1.53 ± 0.13</td>
<td>132.8 ± 9.3</td>
<td>0.219 ± 0.026</td>
<td>0.274 ± 0.024</td>
<td>321.4 ± 6.86</td>
</tr>
</tbody>
</table>

Values are means ± SE of data from 11 vehicle- and 11 capsaicin-treated rats. Minute ventilation (Vₑ), tidal volume (Vₜ), breathing frequency (f), inspiratory time (Tᵢ), and expiratory time (Tₑ) were measured in each animal as the mean values over 20-min period immediately preceding O₃ (or air) exposure.
TNF-α, a cryogenic factor (21) and could contribute to the decreases in core body temperature and O₂ consumption observed.

It is not apparent why Vₑ decreases more in the Cap-treated rats, but one possibility is that increased inflammation and injury, caused by loss of protective effects of tachykinins released from the peripheral ends of the neurons, result in increased generation of mediators such as TNF-α. Our laboratory (15) has previously reported that the decreased core body temperature and heart rate that occur in response to O₃ are not different in Cap- and Veh-treated rats, suggesting that metabolic rate decreases to the same extent in both groups.

However, De Sanctis et al. (5) have reported a decreased ventilatory response to hypoxic gas mixtures in Cap-treated rats, probably resulting from loss of tachykinins in the neurons subtending the carotid bodies. It is possible that loss of responsiveness to hypoxia allows the Cap-treated animals to decrease Vₑ to a greater extent than O₂ consumption.

O₃ causes damage to the airway epithelium including vacuolation, sloughing, and necrosis (37), and there is reason to believe that tachykinin effects on the airway epithelium could mediate the protective role of these peptides during O₃ exposure. Baluk et al. (2) reported that 85% of SP-immunoreactive sensory axons in the rat respiratory tract are located in close proximity to the epithelium, and Sertl et al. (34) showed that SP receptors were concentrated in the epithelium of the central airways in the rat lung. Tachykinins stimulate epithelial cell migration and proliferation (17). Finally, in cultured bronchial epithelial cells, pretreatment with SP decreases permeability to mannitol, a marker of tight junction permeability. Furthermore, O₂-induced increases in epithelial permeability were attenuated in cells treated with SP (48). These data suggest that SP maintains epithelial cell integrity otherwise damaged by O₃. Weakened epithelial integrity caused by O₃ in the absence of tachykinins could reflect enhanced O₃-induced injury and result in the generation of increased neutrophil chemotactic factors.

Tachykinins also have effects on airway blood flow and mucous secretion that may protect the airways from O₃-induced damage. In rat airways, injection of SP increases microvascular blood flow through NK₁ receptors (29), and mucosal application of SP stimulates mucous secretion in an isolated trachea preparation (46). Increased blood flow to airway mucosa could facilitate removal of O₃ from the airway surface, and increased mucous secretion could contribute to the protection by covering the epithelium and reducing the concentration of O₃ that actually reaches the lung surface.

Exposure to O₃ (1 ppm for 3 h) did not cause any change in airway responsiveness to inhaled aerosolized methacholine in Veh (control)-treated rats (Table 2). These results are similar to reports by our laboratory and others of rats of the same strain exposed to the same concentrations of O₃ (15, 39) and measured at the same point in time after O₃ exposure, although airway hyperresponsiveness can be induced in rats with higher concentrations of O₃ or in other strains (9, 42). However, our laboratory (15) has previously reported that, even though O₃ exposure did not induce an increase in airway responsiveness in Veh-treated rats, the same concentration and time of O₃ exposure did increase responsiveness in Cap-treated rats. In contrast, combined inhibition of NK₁ and NK₂ receptors throughout O₃ exposure and methacholine challenge did not result in airway hyperresponsiveness (Table 2). These results suggest that tachykinins per se are not involved in the development of O₃-induced hyperresponsiveness in rats, even though C fibers are. It is unlikely that the lack of effect of the TKRAs on airway hyperresponsiveness

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**Fig. 1. Effects of exposure to filtered air on minute ventilation (Vₑ).** Results are means ± SE of data from 3 vehicle (Veh)-treated (○) and 3 capsaicin (Cap)-treated (●) rats and are expressed as % changes from baseline values. Time 0, time at which ozone would have been introduced into the chamber in ozone-exposed animals, but in these animals ozone was never introduced, and animals breathed only filtered air in the chamber. Baseline values are mean values in 20-min period before time 0.

O₃ in rats has not been evaluated, but in awake dogs O₃ exposure still results in decreases in Vₑ and Tₑ, even in dogs with both cervical vagi cooled to 0°C (31), suggesting that nonvagal mechanisms may be important. For example, it is possible that decreases in Vₑ evoked by O₃ are the result of changes in metabolic rate. Both our laboratory and others have reported a decrease in O₂ consumption, core body temperature, and heart rate during O₃ exposure in rats (15, 25, 32). Furthermore, O₃ exposure still results in decreases in VT and TI, even in O₃-exposed animals (40). Vₑ declined fairly slowly after initiation of O₃ exposure (Fig. 2). In addition, when the O₂ flow into the chamber was turned off, Vₑ remained low for approximately another 25 min before gradually returning to preexposure levels (data not shown). This time course suggests that formation and release of certain chemical mediators may be necessary to evoke changes in Vₑ. For example, O₂ exposure causes increased tumor necrosis factor-α (TNF-α) expression in alveolar macrophages (28). TNF-α is a cryogenic factor (21) and could contribute to the decreases in core body temperature and O₂ consumption observed.

Baluk et al. (2) reported that 85% of SP-immunoreactive sensory axons in the rat respiratory tract are located in close proximity to the epithelium, and Sertl et al. (34) showed that SP receptors were concentrated in the epithelium of the central airways in the rat lung. Tachykinins stimulate epithelial cell migration and proliferation (17). Finally, in cultured bronchial epithelial cells, pretreatment with SP decreases permeability to mannitol, a marker of tight junction permeability. Furthermore, O₂-induced increases in epithelial permeability were attenuated in cells treated with SP (48). These data suggest that SP maintains epithelial cell integrity otherwise damaged by O₃. Weakened epithelial integrity caused by O₃ in the absence of tachykinins could reflect enhanced O₃-induced injury and result in the generation of increased neutrophil chemotactic factors.
reflects inadequate receptor antagonism. Treatment with CP-99994 and SR-48968 caused a log shift in the dose of SP required to elicit a change in blood pressure (Table 3). The observation that TKRA treatment also altered the BAL cell profile (Table 1) further supports the efficacy of the receptor antagonists. One possible explanation for the augmentation of airway responsiveness by Cap pretreatment but not by tachykinin-receptor inhibition is that substances other than tachykinins present in C fibers are important. Possible candidates are calcitonin gene-related peptide and nitric oxide (NO). Both are colocalized with tachykinins in C fibers and act as bronchodilators (3, 7, 11). Absence of these potent bronchodilators coreleased from C-fiber endings on O₃ stimulation might be responsible for augmented airway responsiveness in Cap-pretreated rats.

There were also differences between TKRA treatment and neonatal Cap treatment in the effects on O₃-induced BAL protein changes. BAL protein concentration was significantly increased in O₃-exposed, Cap-treated rats compared with Veh-treated rats, but TKRAs did not alter the effects of O₃ on BAL protein, despite significant increases in BAL neutrophils (Table 1). These results suggest that substances other than tachykinins present in C fibers may act to counteract the O₃-induced changes in vascular permeability that lead to changes in protein. NO has been reported to maintain pulmonary vascular endothelial integrity (11, 20), and, in guinea pig airways, inhibition of endogenous NO resulted in enhancement of vagally induced plasma albumin leakage (20). Lack of the endogenous NO present in C-fiber endings may thus contribute to the increased plasma protein leakage induced by O₃ exposure in Cap-treated animals.

There is evidence of species differences in the effect of C fibers on O₃-induced airway responsiveness. In guinea pigs, depletion of neuropeptides by Cap treatment attenuates O₃-induced airway hyperresponsiveness (38). In rats, Cap pretreatment promotes airway hyperresponsiveness after O₃ exposure (15). One potential explanation for this species difference is the differing bronchoconstrictor potency of tachykinins. In guinea pigs, SP and NKA are both potent bronchoconstrictors (19), whereas, in rats, depending on the strain, the tachykinins either have a weak constrictor potential (16) or actually cause dilation (6). In contrast, effects of Cap pretreatment on lung injury induced by O₃ appear
to be similar in the two species. Both in this study in rats (Table 4) and in the study of Tepper et al. (38) in guinea pigs, increased protein in BAL fluid was observed in Cap-treated animals compared with Veh-treated controls. Similar results were obtained by Sterner-Kock et al. (37). The accumulation of protein in BAL has been shown to be a sensitive indicator of O₃-induced pulmonary damage (43). Hence, our data and those of others support the hypothesis that C fibers act to attenuate O₃-induced pulmonary damage, at least in these two species. Similarly, evidence for a role for C fibers in inhibiting the development of airway injury and/or inflammation has also been obtained with other inhaled irritants. More severe lung injury and/or inflammation in the airways has been observed in C-fiber-ablated rats after chronic sulfur dioxide (22, 23) or hydrogen sulfide (30) exposure, and in Cap-treated guinea pigs after acute acrolein inhalation (41).

In summary, our results show that, in rats, more neutrophils are recovered by BAL when tachykinin-receptor antagonists are administered throughout O₃ exposure. These results are consistent with observations reported herein and with other reports of greater inflammation after O₃ exposure in Cap-treated rats lacking C fibers, compared with Veh-treated controls (37). The greater inflammatory response does not appear to be the result of a greater inhaled dose of O₃, because Vₑ was decreased in Cap-treated rats relative to Veh-treated controls during O₃ exposure. Together, the results support the hypothesis that tachykinins released from peripheral ending of C fibers mediate at least part of the protective effects of these afferents against O₃-induced pulmonary inflammation.

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