Substance P released from intrinsic airway neurons contributes to ozone-enhanced airway hyperresponsiveness in ferret trachea

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Ozone (O₃), a chemical irritant associated with air pollution, has been shown to increase specific airway resistance in human subjects (20) and induce airway hyperresponsiveness (AHR) and neutrophilic inflammation in a variety of animal models, including rats, dogs, and guinea pigs (23, 25, 26, 38). Substance P (SP) levels in human airway lavage fluid are increased after O₃ exposures (18), and several studies demonstrate that O₃ activates bronchial C fibers, which store and release SP (9, 43). SP released from excitatory nonadrenergic noncholinergic sensory nerves has been linked to airway hyperresponsiveness (39). Early studies demonstrated that SP produces smooth muscle contraction and increases vascular permeability in guinea pig airway (29). Although SP has a direct effect on smooth muscle contractility and permeability in some species, it has no direct effect on tracheal smooth muscle responsiveness in ferret trachea, and all neurally mediated contraction in ferret trachea is cholinergic (47). However, SP also acts as a neuromodulator increasing cholinergic sensitivity of airway smooth muscle (6) and increasing the release of acetylcholine (ACh) from cholinergic neurons in the airways (36), either of which could affect airway smooth muscle responsiveness.

Although SP is generally considered a sensory neuropeptide in the airways, it is also synthesized in the parasympathetic neurons of intrinsic airway ganglia of the ferret trachea (11, 13). However, the possibility that airway neurons release SP during O₃ exposure and their contribution to AHR has not been examined. Our laboratory reported previously that in vivo O₃ exposure enhanced airway responsiveness to cholinergic agonist and to electrical field stimulation (EFS) and that a neurokinin (NK) 1-receptor antagonist partially abolished this response (47). The response persisted throughout a culture period intended to deplete the airway of extrinsic, sensory innervation. The findings suggested that O₃-induced AHR is partially mediated through SP released from neurons in airway ganglia. However, these experiments did not entirely eliminate the possibility that the hyperresponsiveness observed after culture may have resulted from the persistent effect of SP released by sensory nerve fibers that were viable during the exposure period or in the early hours of the culture period. To address this problem, we developed a method for in vitro O₃ exposure. This approach would allow O₃ exposure of tracheal segments that had been cultured for 24 h, a procedure that depletes airways of extrinsic innervation, including sensory nerves (15, 49). AHR was assessed by measuring tracheal smooth muscle responses to

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methacholine (MCh), which reflects tracheal smooth muscle sensitivity to cholinergic agonist, and to EFS, which evaluates tracheal smooth muscle responses resulting from the release of ACh from airway cholinergic nerves. The purpose of this study was to determine the role of SP in mediating response to in vitro O₃ exposure after depletion of sensory nerves enhances smooth muscle responsiveness in ferret trachea. We hypothesize that O₃ exposure increases the SP levels in parasympathetic neurons of intrinsic tracheal ganglia and that enhanced SP release increases smooth muscle contractility either directly, by increasing cholinergic contractility of tracheal smooth muscle, or indirectly, by enhancing ACh release from cholinergic nerve terminals innervating tracheal smooth muscle.

**METHODS**

Female, nonalbino ferrets (Marshall Farms, North Rose, NY), weighing 250–500 g, were housed two to four per cage with access to food and water ad libitum in an American Association for Accreditation of Laboratory Animal Care-accredited facility. All procedures were performed in accordance with the recommendations of the Guide for the Care and Use of Laboratory Animals, published by the National Institutes of Health, and were also approved by the West Virginia University Animal Care and Use Committee.

Materials. ACh chloride, MCh chloride, atropine sulfate, hydrocortisone hemisuccinate, amphotericin B, and recrystallized bovine insulin were obtained from Sigma Chemical (St. Louis, MO). Penicillin G, streptomycin, fetal calf serum, and CMRL 1066 were obtained from Gibco (Grand Island, NY). CP-99994 was obtained from Pfizer (Groton, CT). SP antibody was obtained from Pensinula (Belmont, CA). Fluorescein isothiocyanate-labeled goat anti-rabbit antibody was obtained from ICN Immunobiologicals (Costa Mesa, CA).

Organotypic cultures of ferret trachea. Organotypic cultures of tracheas from normal ferrets were used following a modification of our laboratory’s previously described technique (15). Under sterile conditions, tracheas were removed and washed with cold culture medium (described below). The tissue was then placed in a petri dish with culture medium and cut into 60-mm-long segments beginning at the carina. After a second wash, the segments were placed directly on the bottom of petri dishes containing fresh culture medium. In some experiments, capsaicin (10⁻³ M), which depletes SP from sensory nerve terminals (16, 30, 45), or the vehicles of capsaicin, were added to the culture medium and maintained throughout the experiment to determine the role of SP in airway neurons. The culture medium consisted of CMRL 1066 containing 0.1 μg/ml hydrocortisone hemisuccinate, 1 μg/ml recrystallized bovine insulin, 60 μg/ml penicillin G (100 units/ml), 10 μg/ml amphotericin B, 100 μg/ml streptomycin, and 5% heat-inactivated fetal calf serum. The petri dishes were then placed in a controlled-atmosphere culture chamber and gassed with 95% O₂-5% CO₂. The chamber was placed on a rocker and incubated at 37°C for 24 h. After culture, smooth muscle responses were measured in the segments.

In vitro O₃ exposure. The tracheal segments were mounted vertically in Krebs solution, securely tied to upper and lower hose connectors, and stretched to normal resting tension in a glass exposure vessel. The upper hose connector was connected to the mixing chamber, which is a Plexiglas cylinder used to mix the incoming humidified air and O₃ streams before they are administered to the tracheal lumen. O₃ was produced by an O₃ generator (ENMET, Blairsville, PA) that utilizes a low-pressure mercury vapor lamp with high-output ultraviolet radiation of 254-nm wavelength producing up to 150 parts/million (ppm) of O₃ at 1 l/min (0.294 mg l⁻¹) with ambient air as the input gas. To avoid decomposition of O₃, all the tubes exposed to O₃ were made of glass or Teflon. Humidified air was generated and regulated by a humidifier (Electro-Tech Systems, Glenside, PA), a relative humidity controller (Electro-Tech System), and peristaltic pump (Barnant, Barrington, IL), which work in conjunction to maintain a set range of percent relative humidity as well as a constant downstream pressure to prevent backflow of O₃ into the humidifier assembly. The relative humidity controller was set at 79%. The peristaltic pump speed was set to allow mixing of humidified air into the mixing chamber, resulting in an O₃ concentration of 2 ppm. The lower hose connector was connected to an O₃ analyzer (model OA 350-2R, Forney, Carrollton, TX) that continuously measured the O₃ concentration in the tracheal lumen.

All in vitro O₃ exposures were done at 2 ppm for 1 h. A separate group of tracheas was subjected to air exposure in which procedures identical to those described above were followed, except that O₃ was not delivered to the mixing chamber.

Measurement of smooth muscle contraction in vitro. Tracheal smooth muscle reactivity was evaluated by measuring contractile responses to MCh or EFS. MCh responses measure smooth muscle responses to the applied agonist, whereas EFS evaluates tracheal smooth muscle responses resulting from the release of ACh from airway nerves. The segments from air- or O₃-exposed tracheas were cut into 3-mm-wide strips, mounted in holders, and maintained in gassed (95% O₂-5% CO₂) modified Krebs-Henseleit (MKH) solution at 37°C with a composition (in mM) of 113 NaCl, 113, 4.8 KCl, 2.5 CaCl₂, 1.2 MgSO₄, 24 NaHCO₃, 1.2 KH₂PO₄, and 5.7 glucose, pH 7.4. The strips were tied at each end with 4-0 silk and connected to separate strips by adding a series of concentrations of MCh to the MKH solution in the baths every 10 min. About 1 h after equilibration, cumulative concentration-response curves were completed, and resting tension was back to baseline, EFS-induced responses were measured in the segments. When the response curves were completed, strips were washed by changing the MKH solution in the baths every 10 min. About 1 h after concentration-response curves were completed and resting tension was back to baseline, EFS-induced responses were obtained with a Grass S48 stimulator (Grass Instruments, West Warwick, RI). Frequency-response curves were constructed by increasing the frequency from 1 to 30 Hz by using a submaximum voltage of 120 V, 0.2-ms pulse duration, and 10-s train duration. Between each stimulation period, 10 min were allowed for the previous response to return to baseline. EFS-induced contractions were normalized as a percentage of the response to 10⁻² M ACh (%ACh response). In some experiments, nicotine (10⁻⁶ M) was added to the Krebs solution to verify that the responses elicited by EFS were mediated exclusively by the release of ACh from cholinergic nerves.

**Immunocytochemistry.** Immunocytochemical procedures for localizing neuropeptides in neurons and nerve fibers are
identical to those described previously (11, 12). Briefly, the airways were removed 1 h after the end of the O₃ or air exposure, immediately fixed in picric acid-formaldehyde fixative for 3 h, and rinsed three times with a 0.1 M phosphate-buffered saline containing 0.3% Triton X-100. Then the airways were frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at −80°C. The tracheae were frozen on cork supports and oriented with the dorsal surface uppermost so that the tracheal muscle would be sectioned in a coronal plane. Cryostat sections (12-μm thickness) were collected on gelatin-coated coverslips and dried briefly at room temperature, covered with rabbit anti-SP antibody diluted 1:200, incubated in a humid chamber at 37°C for 30 min, and rinsed three times with a solution of 1% bovine serum albumin-phosphate-buffered saline and Triton X-100, with 5 min allowed for each rinse. The sections were then covered with fluorescein isothiocyanate-labeled goat anti-rabbit antibody diluted 1:100, incubated at 37°C for 30 min, and rinsed. After all immunocytochemical procedures were conducted, the coverslips were mounted with Fluoromount and observed with a fluorescence microscope equipped with fluorescein (excitation wavelengths from 455 to 500 nm, and emission wavelengths >510 nm). Controls consisted of testing the specificity of primary antiserum by absorption with 1 μg/ml of the specific antigen. Nonspecific background labeling was determined by omission of primary antiserum.

To measure fluorescence intensity in longitudinal trunk (LT) neurons, images were digitally recorded by using an AX 70 microscope (Olympus America, Melville, NY) with the SPOT 2 digital camera (Diagnostics Instruments, Sterling Heights, MI). Fluorescence intensity of SP was measured by using commercial image-processing software (Optimas 6.5, Media Cybernetics, Silver Spring, MD). The intensity recordings were calibrated by using the InSpeck Green (505/515) microscope image intensity calibration kit (Molecular Probes, Eugene, OR). The LT neurons were identified by drawing the perimeter of the cell, and the fluorescence intensity was reported as gray level for each neuron. Neurons with a gray level <50 were considered negative because they were at or below the general background. Fluorescence intensities of ≥50 were counted as labeled neurons. To measure SP innervation of superficial muscular plexus (SMP) neurons, all identifiable vasoactive intestinal peptide (VIP)-positive neurons [which has been shown to label >90% of all these neurons (11)] were subjectively scored as either innervated or not innervated on the basis of the occurrence of SP in variabilities in apparent direct contact with cell bodies. All identifiable LT and SMP neurons were evaluated in every fifth section collected from serial sections, usually amounting to a total of 10–15 sections analyzed.

For measuring nerve fiber density in tracheal smooth muscle, images of SP-containing nerve fibers were collected in series by using the Zeiss LSM 510 confocal microscope. A series of images representing all of the tracheal smooth muscle in a section were collected in digital files, saved to an internal database, and measured with Optimas software. Regions of smooth muscle were selected by using the rhodamine channel to avoid possible bias created by the presence or absence of nerve fibers. The smooth muscle regions were outlined to measure total cross-sectional area of smooth muscle. The microscope was then switched to reveal nerve fibers in the fluorescein channel and the image digitally captured. The proportion of nerve fibers was determined by segmenting using threshold gray levels with the Optimas software. Then, the percentage of nerve fiber density was calculated as the percentage of total cross-sectional area of smooth muscle occupied by SP-immunoreactive nerve fibers. At least 10 measurements were made for each section, and 15 sections were measured in each animal.

Data analysis. Unless otherwise stated, results are expressed as means ± SE. Contractions elicited by EFS were expressed as a percentage of the maximal contraction elicited by Mch. Contractions to Mch were normalized as percentage of the respective maximal responses for each agonist. The half-maximum concentration (EC₅₀) for Mch was calculated by using a four-parameter logistic curve fit (Sigmoidal, SigmaPlot 2000). The 95% confidence interval was also calculated. Force development was expressed by normalizing force (g) divided by the wet weight of the tissue. LT neurons were expressed as a percentage of SP-positive cell bodies, and SMP neurons were expressed as a percentage of SP-innervated cell bodies. Nerve fiber density was expressed as a percentage of the area of SP-immunoreactive nerve fibers in the total area of the smooth muscle. Statistical analysis of

Table 1. Effect of O₃ on cumulative concentration-response curves for MCh in noncultured tracheal smooth muscle

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>O₃</th>
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<tbody>
<tr>
<td></td>
<td>EC₅₀, M</td>
<td>T_max, g tension/g tissue</td>
</tr>
<tr>
<td>MCh responses</td>
<td>3.46 × 10⁻⁷ (1.02–5.90)</td>
<td>111.6 ± 16.1</td>
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</table>

Values are means ± SE with 95% confidence interval in parentheses. T_max, maximum tension; EC₅₀, half-maximum concentration; MCh, methacholine. *Significant difference between air (control) and ozone (O₃) exposure, P ≤ 0.05.
immunocytochemistry, EC$_{50}$, and EFS was performed by using one- or two-way repeated-measures ANOVA. When the main effect was considered significant at $P < 0.05$, pairwise comparisons were made with a post hoc analysis (Fisher’s least significant difference). A value of $P < 0.05$ was considered significant, and $n$ represents the number of animals studied.

RESULTS

Study 1: Effect of in vitro O$_3$ exposure on airway responsiveness in noncultured and cultured tracheas. The initial experiments examined the effect of in vitro O$_3$ exposure on noncultured tracheas. The cumulative dose-response curve for Mch was markedly shifted to the left after exposure to O$_3$ (Fig. 1A), and the EC$_{50}$ value for Mch (Table 1) was decreased by 66% in O$_3$-exposed tracheal segments. Exposure to O$_3$ also increased the smooth muscle response to EFS. A leftward shift in the frequency-response curve to EFS was observed after O$_3$ exposure (Fig. 1B), and contractions produced by EFS at 10 and 30 Hz were significantly increased by 33 and 26%, respectively, after O$_3$ exposure ($P < 0.05$).

The next studies were done to examine the contribution of intrinsic neurons by measuring the O$_3$-enhanced AHR of cultured tracheal segments. Previous studies have shown that innervation of smooth muscle by airway neurons remains intact during short-term culture and that SP-containing sensory neurons mostly degenerate (15, 49). Therefore, tracheal segments were maintained in organotypic culture for 24 h and then exposed to 2 ppm O$_3$ for 1 h in the in vitro exposure chamber. The cumulative concentration-response curve for Mch (Fig. 2A) was shifted to the left and EC$_{50}$ value (Table 2) for Mch was decreased by 66% in tracheal strips after O$_3$ exposure. Contractions produced by EFS at 10 and 30 Hz were significantly increased by 29 and 30%, respectively, in tracheal strips after O$_3$ exposure (Fig. 2B). The contractions to Mch and EFS in both noncultured and cultured tracheal segments were totally abolished after treatment with $10^{-6}$ M atropine (Figs. 1 and 2).

Previous studies have indicated that pretreatment with a high concentration of capsaicin depletes SP in sensory neurons (16, 49). The next studies were done to confirm that O$_3$-exposed cultured tracheal segments lacked SP-containing sensory nerves. The rationale was that application of $10^{-5}$ M capsaicin should not affect O$_3$-enhanced smooth muscle responses if the segments were initially depleted of sensory nerves. Even in the presence of capsaicin, the cumulative concentration-response curve for Mch (Fig. 3A) was shifted to the left and the EC$_{50}$ value (Table 3) for Mch was decreased by 61% after O$_3$ exposure. Contractions produced by EFS at 10 and 30 Hz were significantly increased by 36 and 33%, respectively, after O$_3$ exposure and were unaltered by capsaicin (Fig. 3B). These findings support our assumption that SP-containing nerve fibers originating from sensory neurons are depleted after culture.

Study 2: Effects of in vitro O$_3$ exposure on SP release from in airway neurons. The next experiments examined the role of SP in O$_3$-enhanced airway responsiveness by blocking the NK$_1$ receptor. Cumulative concentration-response curve for Mch and the EFS-stimulated contractions at 10 and 30 Hz demonstrated expected changes in cultured tracheal segments after O$_3$ exposure in the group pretreated with saline (Fig. 4, A and C, and Table 4). Mch contractions after O$_3$ exposure were still significantly increased subsequent to the NK$_1$ antagonist (Fig. 4B and Table 4). However, the O$_3$-enhanced contractile responses to EFS were not different from air-exposed controls in tracheas treated with the NK$_1$ antagonist prior to O$_3$ exposure (Fig. 4D). EFS-stimulated airway contractions at 10 and 30 Hz increased only by 10 and 7%, respectively, after O$_3$ in tracheas pretreated with CP-99994, compared with 27 and 33% in tracheas pretreated with saline (Table 4).

Table 2. Effect of O$_3$ on cumulative concentration-response curves for Mch in organotypic cultured tracheal smooth muscle

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>O$_3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>EC$_{50}$, M</td>
<td>$1.87 \times 10^{-6}$ (1.09–2.65)</td>
<td>$6.39 \times 10^{-7}$ (3.25–9.53)</td>
</tr>
<tr>
<td>g tension/g tissue</td>
<td>$101.1 \pm 13.6$</td>
<td>$99.2 \pm 14.6$</td>
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Values are means ± SE with 95% confidence interval in parentheses. *Significant difference between air (control) and O$_3$ exposure, $P < 0.05$.

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Study 3: Changes in immunoreactive SP-containing neurons of intrinsic airway ganglia. These studies examined the effect of O3 on SP-containing cell bodies in LT and SP innervation in cell bodies of the SMP in cultured trachea. Characterization of LT and SMP ganglia has been described previously (11). About 36% of the LT cell bodies labeled for SP (Figs. 5A and 6A). After exposure to O3, nearly 56% of the cell bodies in the LT contained SP (Figs. 5B and 6A). In the SMP, ~23% of the neurons were innervated by SP-containing nerve fibers in control tracheas (Figs. 5C and 6B) but nearly 46% were innervated by SP-containing nerve fibers after O3 exposure (Figs. 5D and 6B). SP nerve fiber density from the tracheal smooth muscle was significantly increased from 0.33% in controls to 0.47% after exposure to O3 (Figs. 5, E and F, and 6C). This represents a 45% increase in nerve fiber density.

DISCUSSION

This study shows that in vitro O3 exposure enhances tracheal smooth muscle responsiveness in the ferret, as evidenced by elevated contractility to MCh and EFS. O3-induced AHR was elicited even in tracheal segments cultured for 24 h, a procedure shown to cause a significant anatomic and functional loss of SP-containing sensory fibers while maintaining viability of intrinsic airway neurons (15, 49). The efficacy of the depletion was further verified in the present experiments by showing that the O3-induced responses were not altered by application of capsaicin in cultured tracheal segments. These experiments support the conclusion that the O3-enhanced tracheal smooth muscle responsiveness did not result from sensory nerve fibers that may have survived after culture. Instead, the findings suggest that parasympathetic neurons of intrinsic airway ganglia contribute to the increased smooth muscle responsiveness induced by in vitro O3 exposure. The elevation of airway smooth muscle responses by in vitro O3 exposure was attenuated by treatment with a NK1-receptor antagonist, indicating that SP release played a key role in the mechanism responsible for the enhancement of smooth muscle contractile responses. The finding that blocking NK1 receptors reduced EFS-induced atropine-sensitive contractions but did not alter MCh sensitivity suggests that the SP released during O3 exposure increases ACh release from cholinergic nerve terminals. The observations that O3 exposure increased the proportion of neurons in the LT expressing SP, the SP innervation of SMP neurons, and the SP innervation of tracheal smooth muscle in cultured trachea all support the conclusion that O3 exposure elevates endogenous SP levels in parasympathetic neurons of airway ganglia.

SP localized in the peripheral endings of nerves innervating the lung and airways originates in nerve cell bodies located both in sensory (12, 21) and intrinsic airway (10, 11, 13, 15, 27) ganglia. Stimulation of sensory nerve afferents by inhalation of irritants is known to trigger the release of neuropeptides from afferent endings (28, 31, 46, 48). However, much of the evidence implicating SP as a mediator of altered airway responsiveness does not differentiate between sensory and intrinsic airway neurons as the source of SP. The finding that the NK1-receptor antagonist CP-99994 significantly attenuates the effect of O3 on EFS-stimulated contractile responses in cultured trachea implicates the involvement of SP as the mediator of O3 action on airway smooth muscle. Although SP is a known bronchoconstrictor in guinea pig airway (3, 29), direct action of SP on smooth muscle does not appear to be an important effect in the ferret trachea because all of the smooth muscle contractile effects of O3 were atropine sensitive. Thus the logical explanation of the O3 effect in ferret is that O3 alters tracheal smooth muscle responsiveness to EFS by increasing the production and release of SP from intrinsic tracheal neurons. Previous studies have shown that SP enhances tracheal smooth muscle responsiveness by enhancing ACh release from parasympathetic nerve terminals (34, 41, 44).

Table 3. Effect of capsaicin on cumulative concentration-response curves for MCh in organotypic cultured tracheal smooth muscle after in vitro O3 exposure

<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>O2</th>
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<tbody>
<tr>
<td>MCh responses</td>
<td>2.06 × 10⁻⁶ (1.39–2.73)</td>
<td>7.99 × 10⁻⁷ (5.24–10.74)</td>
</tr>
<tr>
<td>EC₅₀, M</td>
<td>94.5 ± 12.9</td>
<td>92.6 ± 13.2</td>
</tr>
<tr>
<td>T₉₀, g tension/g tissue</td>
<td>92.6 ± 13.2</td>
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Values are means ± SE with 95% confidence interval in parentheses. *Significant difference between air (control) and O3 exposure, P < 0.05.

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activating the inhibitory nonadrenergic noncholinergic
(iNANC) neurons to release bronchodilator transmitters
NO and VIP. This arrangement may serve to bal-
ance the constrictor actions of ACh at airway gan-
glia. However, after O₃ exposure, the levels of SP
production in LT neurons increases. This increase
correlates with an increase in innervation at the SMP
neurons and supports the possibility that SP release at
SMP neurons may also be increased by O₃ exposure.
The effect of increased SP release at SMP neurons may
serve to downregulate the iNANC neurons, reducing
VIP and NO release at smooth muscle. Such an effect
would be consistent with enhanced tracheal smooth
muscle contraction and AHR. A few studies have asso-
ciated the attenuation of iNANC innervation with
asthma or increased smooth muscle responsiveness. In
humans, VIP nerve fiber density is reduced in individ-
uals with severe asthma (37). Physiological studies
have demonstrated reduced iNANC activity in aller-
gen-sensitized and -challenged rabbits (17) and in
young ferrets after viral infection (8). Although the
effects of SP on iNANC neurons is not known, activa-
tion of NK₃ receptors in guinea pig airway neurons
inhibits excitability and facilitates synaptic trans-
mission in cholinergic neurons (5, 35).

A role for inflammatory mediators should also be
considered. Our laboratory’s previous study showed
increased innervation of SMP after interleukin-1β
treatment (49). The parallels with the findings in the
present paper may suggest that the effects of O₃ are
mediated through the generation of inflammatory me-
diators. Thus the initiation of an inflammatory process
by any irritant may eventually converge on a common
pathway that involves upregulation of SP levels of
airway neurons projecting to and modulating both air-
way smooth muscle and other neurons in the airway
neuronal plexus.

Plasticity of intrinsic airway neurons is certainly not
the only mechanism responsible for O₃-induced re-
sponses and smooth muscle hyperresponsiveness.
Many studies have demonstrated the important role
of C-fiber activation of central vagal reflexes in mediating
rapid shallow breathing, tachypnea, and bronchocon-
striction after O₃ exposure (40) as well as causing
smooth muscle hyperresponsiveness (26). In addition
to generating central reflexes, C-fiber activation ini-
tiates local axon reflexes within the airway wall, re-
sulting in the release of SP from collateral afferent
nerve fibers near smooth muscle to produce smooth

Table 4. Effect of CP-99994 on cumulative concentration-response curves for MCh in organotypic cultured
tracheal smooth muscle after in vitro O₃ exposure

<table>
<thead>
<tr>
<th>MCh</th>
<th>EC₅₀, M</th>
<th>Tₘₐₓ, g tension/g tissue</th>
<th>EC₅₀, M</th>
<th>Tₘₐₓ, g tension/g tissue</th>
</tr>
</thead>
<tbody>
<tr>
<td>Saline</td>
<td>1.45 × 10⁻⁶ (0.91–1.99)</td>
<td>106.4 ± 13.3</td>
<td>5.30 × 10⁻⁶ (2.05–8.55)</td>
<td>104.2 ± 14.1</td>
</tr>
<tr>
<td>CP-99994</td>
<td>1.51 × 10⁻⁶ (1.02–2.00)</td>
<td>103.5 ± 12.4</td>
<td>9.48 × 10⁻⁶ (6.04–12.92)</td>
<td>101.8 ± 14.6</td>
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</table>

Values are means ± SE with 95% confidence interval in parentheses. *Significant difference between air (control) and O₃ exposure, P ≤ 0.05.
muscle hyperresponsiveness (22, 25). The release of local inflammatory mediators, such as arachidonic acid metabolites (19, 24) and cytokines (42, 49), may also increase airway smooth muscle responsiveness to O₃ exposure. Our findings identify the intrinsic airway neurons as another regulatory mechanism in what appears to be a highly redundant system designed to protect the distal airways and gas-exchange regions of the lung from inhaled irritants, including O₃. However, because these neurons are known to project to cholinergic neurons (50), the unique role of intrinsic neurons may be to provide prejunctional modulation of ACh release from cholinergic nerve endings innervating airway smooth muscle.

In conclusion, our results show that in vitro O₃ exposure increases SP levels in nerve cell bodies and fibers of intrinsic airway nerves innervating tracheal smooth muscle. At the same time, smooth muscle responses are increased in tracheal segments depleted of sensory innervation. Administration of CP-99994, an antagonist of the NK₁ receptor, attenuates the in vitro O₃ exposure-enhanced tracheal smooth muscle responses to EFS, indicating the enhanced responses is dependent on SP release from intrinsic nerves.

Fig. 5. Fluorescence photomicrographs of substance P (SP)-immunoreactive nerve cell bodies and fibers in longitudinal trunk (LT; A and B) and superficial muscular plexus (SMP; C and D) and SP-immunoreactive nerve fiber density (NFD) in tracheal smooth muscle (E and F) after in vitro exposure to air (control) or ozone. A: negative SP-immunoreactive LT neurons are seen in the control ganglia. B: most of the LT neurons contain SP immunoreactivity after ozone exposure. C: few SP-immunoreactive cell bodies are present in the SMP of control. D: SP-immunoreactive cell bodies in the SMP are increased after ozone exposure. E: few SP-immunoreactive nerve fibers are present in tracheal smooth muscle of control (NFD of this micrograph is 0.36). F: increased SP-immunoreactive nerve fibers in tracheal smooth muscle after ozone exposure (NFD of this micrograph is 0.53). Magnification: ×285.

Fig. 6. Effects of air (open bars) and ozone (solid bars) exposure on SP-containing nerve cell bodies in LT (A), SP innervation of airway neurons in SMP (B), and SP-immunoreactive (IR) NFD in tracheal smooth muscle (C). Values are means ± SE; n = 6. *Significant difference between air and ozone exposure, P ≤ 0.05.
findings suggest that O₃ exposure increases SP production and release from airway neurons. SP release may contribute to O₃-enhanced smooth muscle responsiveness in ferret trachea by facilitating prejunctional ACh release from cholinergic nerve endings.

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DISCUSSIONS

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