

Role of intrinsic airway neurons in ozone-induced airway hyperresponsiveness in ferret trachea

ZHONG-XIN WU,¹ DAVID F. MAIZE, JR.,^{1,3} BRIAN E. SATTERFIELD,¹ DAVID G. FRAZER,² JEFF S. FEDAN,² AND RICHARD D. DEY¹

¹Department of Anatomy, Robert C. Byrd Health Sciences Center, West Virginia University, Morgantown 26506; ²Health Effects Laboratory Division, National Institute for Occupational Safety and Health, Morgantown, West Virginia 26505; and ³Department of Pharmaceutical Sciences, Nesbitt School of Pharmacy at Wilkes University, Wilkes-Barre, Pennsylvania 18766

Received 29 November 2000; accepted in final form 21 February 2001

Wu, Zhong-Xin, David F. Maize, Jr., Brian E. Satterfield, David G. Frazer, Jeff S. Fedan, and Richard D. Dey. Role of intrinsic airway neurons in ozone-induced airway hyperresponsiveness in ferret trachea. *J Appl Physiol* 91: 371–378, 2001.—Exposure to ozone (O₃) enhances airway responsiveness, which is mediated partly by the release of substance P (SP) from airway neurons. In this study, the role of intrinsic airway neurons in O₃-induced airway responses was examined. Ferrets were exposed to 2 ppm O₃ or air for 1 h. Reactivity of isolated tracheal smooth muscle to cholinergic agonists was significantly increased after O₃ exposure, as were contractions to electrical field stimulation at 10 Hz. Pretreatment with CP-99994, a neurokinin type 1 receptor antagonist, partially abolished the O₃-induced reactivity to cholinergic agonists and electrical field stimulation. The O₃-enhanced airway responses were present in tracheal segments cultured for 24 h, a procedure shown to deplete sensory nerves while maintaining viability of intrinsic airway neurons, and all the enhanced smooth muscle responses were also diminished by CP-99994. Immunocytochemistry showed that the percentage of SP-containing neurons in longitudinal trunk and the percentage of neurons innervated by SP-positive nerve fibers in superficial muscular plexus were significantly increased at 1 h after exposure to O₃. These results suggest that enhanced SP levels in airway ganglia contribute to O₃-induced airway hyperresponsiveness.

airway smooth muscle; sensory nerves; neurokinin receptors; tachykinins; substance P

ACUTE EXPOSURE TO OZONE (O₃), one of the major air pollutants in urban areas, is frequently associated with airway hyperresponsiveness (AHR) (18–20, 34). Although airway smooth muscle contraction is mediated by acetylcholine (ACh) binding to atropine-sensitive M₃-muscarinic receptors (27), separate mechanisms have been proposed as mediating the induction of AHR, including altered M₂ function (39), release of arachidonic acid-derived mediators (4), inhibition of neutral endopeptidase (30), release of tachykinins from sensory nerves (15), and inhibition of release of epithelium-derived relaxing factor (11). Several stud-

ies have shown that increased airway smooth muscle responsiveness after O₃ exposure is mediated by the release of endogenous tachykinins from capsaicin-sensitive nerve fibers in airway wall (18, 19, 34).

Neuropeptides, such as substance P (SP), neurokinin A (NKA), and calcitonin gene-related peptide (CGRP), are localized in the peripheral endings of the nonmyelinated C-fibers innervating the airways and are synthesized in sensory nerve cell bodies located in the jugular and nodose ganglia (17, 21). Other neuropeptides, such as vasoactive intestinal peptide (VIP) and nitric oxide (NO), are associated with neurons of intrinsic airway ganglia located in the adventitia of airway walls (9, 12). However, recent studies have shown that SP may also be present in airway ganglia (6, 8, 14). A substantial portion of the innervation of airway smooth muscle, glands, and blood vessels in the airways originates from neurons with cell bodies in intrinsic airway ganglia (8, 10). Although most innervation of airway epithelium originates from neurons with cell bodies located in nodose or jugular ganglia (7, 17), nerve fibers from cell bodies in intrinsic airway ganglia may also project to the airway epithelium (10), providing a potential neural pathway for local modulation of airway responses.

The role of SP released from neurons in airway ganglia is unknown. Inhalation of chemical irritants triggers the local release of neuropeptides from sensory nerves (22, 37). Neuropeptides, especially SP in the airway, are known to produce potent effects on airway smooth muscle tone (33) and alter neurotransmission through airway ganglia (26, 36). Inhalation of irritants such as antigens (13) and toluene diisocyanate (16) increases SP levels in sensory neurons projecting to the airways. However, whether SP released from intrinsic airway neurons contributes to AHR after inhaled irritants has not been determined. Therefore, the purpose of this study was to investigate the possible involvement of intrinsic airway neurons in the development of AHR induced by O₃ exposure.

Address for reprint requests and other correspondence: R. D. Dey, Dept. of Anatomy, PO Box 9128, West Virginia University, Morgantown, WV 26506 (E-mail: rdey@hsc.wvu.edu).

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METHODS

Female nonalbino ferrets (Marshall Farms, North Rose, NY; 250–500 g body wt) 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. Female ferrets were used, because they adapt better than males to group housing. The ferrets were euthanized by inhalation of 100% CO₂ for 3 min in a sealed exposure chamber. 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.

O₃ exposure. All O₃ exposures were done at 2 ppm in a 12 × 12-in. stainless steel-and-glass chamber for 1 h. O₃ was produced by passing hospital-grade air through a drying and high-efficiency particle filter and then through an ultraviolet light source. The O₃ concentration in the chamber was measured by chemiluminescence with a calibrated O₃ analyzer (model OA 350-2R, Forney, Carrollton, TX) and adjusted every 10 s by an on-line computer. A separate group of animals was subjected to air exposure, in which procedures identical to those described above were followed, except O₃ was not delivered to the mixing chamber. To determine the possible involvement of endogenously released SP, some ferrets were given one injection of CP-99994 (1.0 mg/kg ip), a neurokinin (NK) type 1 (NK₁)-receptor antagonist, 30 min before O₃ or air exposure. The dose of this antagonist was determined on the basis of previous findings (35, 37, 38, 40).

Cultured tracheal segments. To examine intrinsic innervation in tracheas depleted of sensory fibers, organotypic cultures of tracheas were used following a modification of our previously described technique (10). Ferrets were exposed to 2 ppm O₃ or air for 1 h as described above. Immediately after exposure, tracheas were removed and washed with cold culture medium (described below), placed in a petri dish with culture medium, and cut into 10-mm-long segments beginning with the caudal end. After a second wash, the segments were placed directly on the bottom of petri dishes containing fresh culture medium. In some of the cultures, CP-99994 (final concentration 10 mM) was added to the culture media and maintained throughout the experiment to determine the role of SP in intrinsic airway neurons. The antagonist concentration was based on previous studies (1). The petri dishes were then placed in a controlled-atmosphere culture chamber and gassed with 45% O₂-5% CO₂-50% N₂. 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. The culture media was CMRL 1066 containing 0.1 µg/ml hydrocortisone hemisuccinate, 1 µg/ml recrystallized bovine insulin, 60 µg/ml penicillin G (100 U/ml), 10 µg/ml amphotericin B, 100 µg/ml streptomycin, and 5% inactivated fetal calf serum.

Measurement of tissue contraction in vitro. Fresh tracheal segments from ferrets 1 h after air or O₃ exposure and from the 24-h cultures were cut into 3-mm-wide strips, mounted in holders, and maintained in gassed (95% O₂-5% CO₂) modified Krebs-Henseleit solution at 37°C with a composition (in mM) as follows: 113 NaCl, 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 positioned between the rings of platinum electrodes in tissue holders. Each holder was anchored in a 10-ml water-jacketed organ bath, and the top string was attached to a force-displacement transducer connected to a recorder (Gould Instruments, Valley View, OH). Strips were equilibrated for 60 min at a

resting tension of 1.0 g, determined in preliminary studies to be optimal for contraction, during which time the modified Krebs-Henseleit solution in the baths was changed every 15 min. After equilibration, cumulative concentration-response curves for ACh and methacholine (MCh) were constructed for separate strips by adding a series of concentrations of ACh or MCh to the bath in half-log-increment concentrations ranging from 10⁻⁹ to 10⁻³ M. The next concentration was not added until the previous response reached a plateau. After concentration-response curves were completed, electrical field stimulation (EFS)-induced responses were obtained with a stimulator (model S48, Grass Instruments, W. Warwick, Richmond, VA). Frequency-response curves were constructed by increasing the frequency from 0.3 to 30 Hz 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 by the percent response of each tissue to 10⁻³ M ACh (%ACh response). In some experiments, atropine (10⁻⁶ M) was added to Krebs solution to verify that the responses elicited by EFS were mediated by the release of ACh from cholinergic neurons. In initial experiments, contractions to EFS in noncultured and cultured tracheal segments were totally abolished after treatment with 10⁻⁶ M atropine (data not shown).

Immunocytochemistry. In a separate group of ferrets exposed using the same O₃ exposure protocol, tracheas were removed 1 h after O₃ or air exposure, fixed in picric acid-formaldehyde fixative for 3 h (32), and rinsed three times with a 0.1 M phosphate-buffered saline containing 0.15% Triton X-100. The tracheas were dissected and frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at -80°C. The tracheas were oriented with the dorsal surface uppermost so the tracheal muscle would be sectioned in a coronal plane.

Cryostat sections (12 µm thick) were collected on gelatin-coated coverslips and dried briefly at room temperature. Immunocytochemical procedures for localizing neuropeptides in neurons and nerve fibers are identical to those described by us previously (6, 7). Briefly, cryostat sections on coated coverslips were covered with SP antibody diluted 1:100, incubated in a humid chamber at 37°C for 30 min, and rinsed with a 1% bovine serum albumin + phosphate-buffered saline containing 0.15% Triton X-100 three times, 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. Then the same procedures were used to label VIP using mouse anti-VIP (1:100) and goat anti-mouse labeled with rhodamine (1:100). VIP labeling was done to allow efficient identification of superficial muscular plexus (SMP) neurons, which are typically difficult to locate but can be easily visualized immunocytochemically, because 90% are VIP immunoreactive. After all immunocytochemical procedures were conducted, the coverslips were mounted with Fluoromount and observed with a fluorescence microscope equipped with fluorescein (excitation wavelengths 455–500 nm, emission wavelengths >510 nm) and rhodamine (excitation 540–504 nm, emission >580 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 using an AX 70 microscope (Olympus America, Melville, NY) with the SPOT 2 (Diagnostics Instruments, Sterling Heights, MI). Fluorescence intensity of SP was measured using commercial image

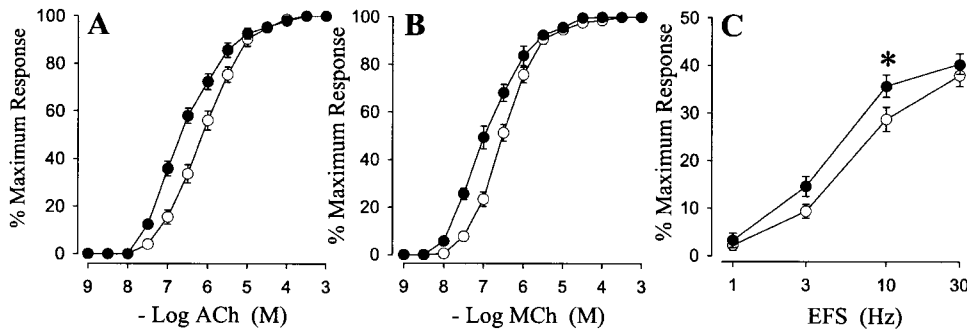


Fig. 1. Cumulative dose-response curve for acetylcholine (ACh, A) and methacholine (MCh, B) and frequency-response curve for electrical field stimulation (EFS, C) in tracheal smooth muscle after exposure to air (○) or 2.0 ppm O₃ (●) for 1 h. Contractions are plotted as percentages of the maximum response. Responses for EFS are plotted as percentages of maximum response to ACh. Values are means $\pm$ SE; $n = 6$. *Significant difference between control and O₃-exposed animals, $P < 0.05$.

processing software (Optimas 6.5, Media Cybernetics, Silver Spring, MD). The intensity recordings were calibrated using the InSpec 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 SMP neurons (identified by VIP colocalization), all identifiable SMP neurons were scored as either innervated or not innervated. All LT and SMP neurons were evaluated in every fifth section collected from serial sections, usually amounting to a total of 10–15 sections analyzed.

Data analysis. Unless otherwise stated, values are means $\pm$ SE. Contractions elicited by EFS are expressed as a percentage of the maximal contraction elicited by ACh. Contractions to ACh and MCh were normalized as a percentage of the respective maximal responses for each agonist. EC₅₀ values for ACh and MCh were calculated using a four-parameter logistic curve fit (Sigmoidal, SigmaPlot 2000) and are presented with 95% confidence intervals. Force development was expressed by normalizing force (g) divided by the wet weight of the tissue. LT neurons are expressed as percent SP-positive cell bodies, and SMP neurons are expressed as percent SP-innervated cell bodies. Statistical analyses of immunocytochemistry and EC₅₀ were performed using Student's *t*-test. Statistical analysis of EFS was performed using two-way repeated-measures analysis of variance. One factor between the groups was O₃ exposure; the other factor within the group was EFS effect. 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). $P < 0.05$ was considered significant, and n represented the number of animals studied.

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 Peninsula (Belmont, CA). Mouse-anti-VIP monoclonal antibody was a gift from Dr. John Porter (University of Texas Southwestern Medical School, Dallas, TX). Fluorescein isothiocyanate-labeled goat anti-rabbit antibody was obtained from ICN Immunobiologicals (Costa Mesa, CA).

RESULTS

Effect of O₃ on airway responsiveness in noncultured trachea. The initial experiments were intended to demonstrate that O₃ exposure increases airway smooth

muscle sensitivity to ACh and MCh. The cumulative dose-response curves for ACh and MCh were markedly shifted to the left after exposure to O₃ (Fig. 1, A and B), and the EC₅₀ values for ACh and MCh (Table 1) were decreased by 69% and 61% in O₃-exposed animals. Exposure to O₃ also increased airway response for EFS. A leftward shift in the frequency-response curve to EFS was observed after O₃ exposure (Fig. 1C), and contraction produced by EFS at 10 Hz was significantly increased by 28% after O₃ exposure.

Effects of NK₁ antagonist in tracheal strips. In separate experiments, NK₁ receptors were blocked with CP-99994 to test the involvement of SP in O₃-induced AHR. In control ferrets pretreated with saline, the dose-response curves and EC₅₀ values for ACh and MCh (Fig. 2, A and C, Table 2) demonstrated the expected changes (Fig. 1) after O₃ exposure: a shift in the cumulative dose-response curves for ACh and MCh to the left of control and a decrease in EC₅₀. A significant increase in EFS-stimulated contraction at 10 Hz was also observed (Fig. 2E). There were no effects of CP-99994 on ACh or MCh dose-response curves obtained from strips in air-exposed animals (Table 2), nor was EFS-induced contraction at 10 Hz affected (Fig. 2F). However, the increase in reactivity to ACh and MCh (Fig. 2, B and D, Table 2) and the potentiated response to 10-Hz EFS stimulation (Fig. 2F) were partly abolished by CP-99994 treatment. EC₅₀ values for ACh and MCh decreased by only 43% and 40% after O₃ exposure and pretreatment with CP-99994 compared with 72% and 66% after pretreatment with saline. In O₃-exposed ferrets, EFS-stimulated airway contraction at 10 Hz increased by 12% after pretreatment with CP-99994 compared with 31% after pre-

Table 1. Effect of O₃ on cumulative dose-response curves for ACh and MCh in trachea smooth muscle

	Control		O ₃	
	EC ₅₀ , M	T _{max} , g/g tissue	EC ₅₀ , M	T _{max} , g/g tissue
ACh	7.45×10^{-7} (4.30–10.60)	115.4 ± 16.3	2.30×10^{-7} * (0.08–4.52)	109.5 ± 11.3
MCh	2.99×10^{-7} (0.50–5.44)	120.8 ± 15.2	1.16×10^{-7} * (0.01–2.31)	113.7 ± 14.1

Values are means $\pm$ SE, with 95% confidence interval in parentheses. EC₅₀, half-maximum concentration; T_{max}, maximum tension; ACh, acetylcholine; MCh, methacholine. *Significantly different from control, $P < 0.05$.

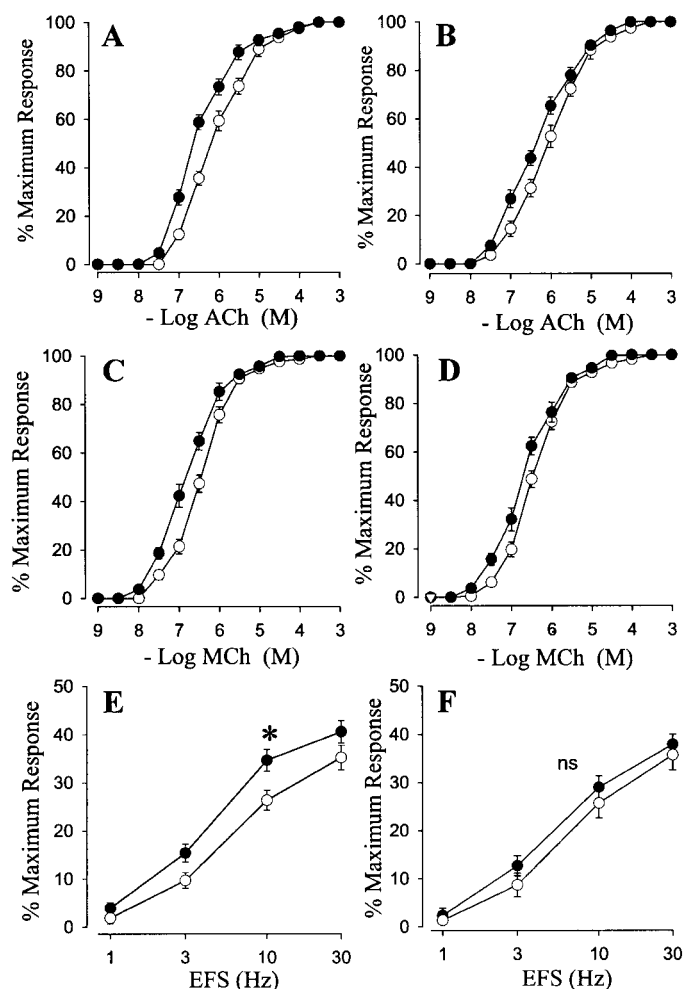


Fig. 2. Effects of saline (A, C, and E) and CP-99994 (B, D, and F) on cumulative dose-response curve for ACh (A and B) and MCh (C and D) and frequency-response curve for EFS (E and F) in tracheal strips from ferrets after exposure to air (○) or 2.0 ppm O₃ (●) for 1 h. Values are means $\pm$ SE; $n = 5$. NS, not significant. *Significant difference between control and O₃-exposed animals, $P < 0.05$.

Table 2. Effect of CP-99994 treatment on cumulative dose-response curves for ACh and MCh

	Control		O ₃	
	EC ₅₀ , M	T _{max} , g/g tissue	EC ₅₀ , M	T _{max} , g/g tissue
<i>ACh responses</i>				
Control	8.04 $\times 10^{-7}$ (5.03–11.05)	106.8 $\pm$ 13.8	2.24 $\times 10^{-7}$ * (0.03–4.18)	101.2 $\pm$ 12.4
CP-99994	9.05 $\times 10^{-7}$ (6.31–11.79)	98.7 $\pm$ 11.2	5.18 $\times 10^{-7}$ (3.33–7.03)	96.4 $\pm$ 10.6
<i>MCh response</i>				
Control	3.14 $\times 10^{-7}$ (1.60–4.68)	112.1 $\pm$ 16.4	1.06 $\times 10^{-7}$ * (0.11–2.01)	108.3 $\pm$ 14.7
CP-99994	3.97 $\times 10^{-7}$ (1.35–6.59)	103.8 $\pm$ 13.8	2.37 $\times 10^{-7}$ (1.55–3.92)	105.7 $\pm$ 14.1

Values are means $\pm$ SE, with 95% confidence interval in parentheses. *Significantly different from control, $P < 0.05$.

treatment with saline. The results indicated that pre-treatment with CP-99994 attenuates O₃-induced responses to ACh, MCh, and EFS.

Effects of NK₁ antagonist in cultured tracheal strips. The next series of studies was done to examine the contribution of SP from intrinsic airway neurons to O₃-induced AHR. Previous studies have shown that innervation of smooth muscle by intrinsic airway neurons remains intact during short-term culture but that SP-containing sensory neurons mostly degenerate (10). Therefore, tracheal segments from ferrets exposed to air or O₃ were maintained in organotypic culture with saline or CP-99994 for 24 h. After culture, dose-response curves for ACh and MCh (Fig. 3, A and C) were shifted to the left, and EC₅₀ values (Table 3) were significantly less in tracheal segments cultured with saline from O₃-exposed ferrets. O₃ exposure also increased EFS-stimulated contractions at 10 and 30 Hz (Fig. 3E) in tracheal segments cultured with saline. However, the increase in reactivity to ACh and MCh

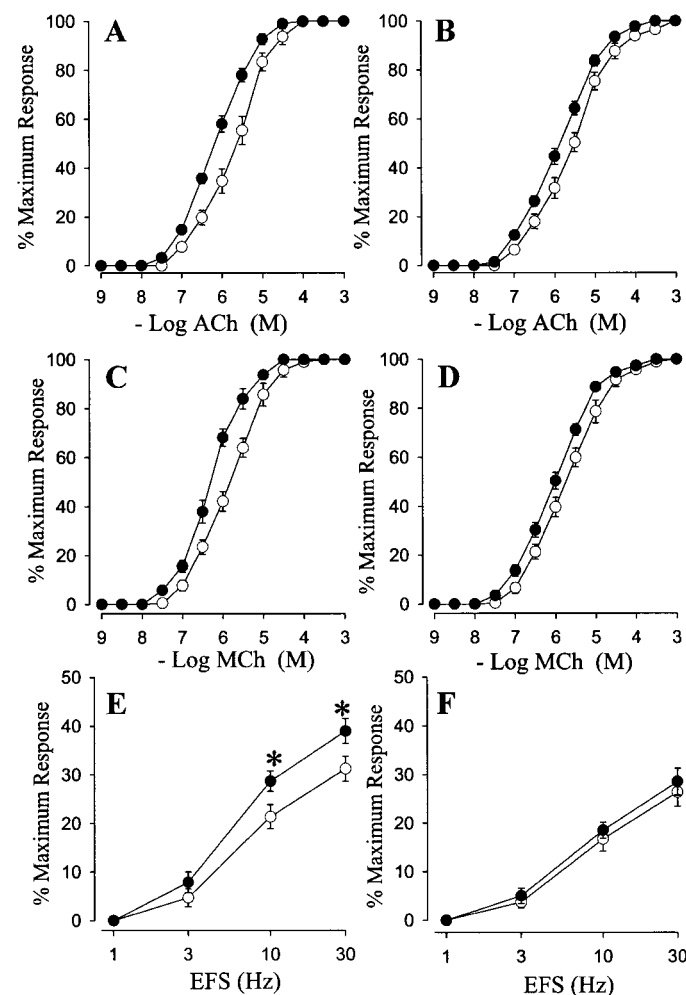


Fig. 3. Effects of saline (A, C, and E) and CP-99994 (B, D, and F) on cumulative dose-response curve for ACh (A and B) and MCh (C and D) and frequency-response curve for EFS (E and F) in cultured tracheal strips from ferrets after exposure to air (○) or 2.0 ppm O₃ (●) for 1 h. Values are means $\pm$ SE; $n = 6$. *Significant difference between control and O₃-exposed animals, $P < 0.05$.

Table 3. Effect of CP-99994 treatment on cumulative dose-response curves for ACh and MCh in cultured tracheal strips

	Control		O ₃	
	EC ₅₀ , M	T _{max} , g/g tissue	EC ₅₀ , M	T _{max} , g/g tissue
<i>ACh responses</i>				
Control	2.86 × 10 ⁻⁶ (1.04–4.68)	96.4 ± 11.4	7.17 × 10 ^{-7*} (3.64–11.70)	95.5 ± 10.8
CP-99994	3.02 × 10 ⁻⁶ (1.22–4.82)	93.2 ± 10.5	1.58 × 10 ⁻⁶ (0.21–2.95)	94.1 ± 12.1
<i>MCh responses</i>				
Control	1.89 × 10 ⁻⁶ (0.73–3.05)	98.8 ± 10.5	4.34 × 10 ^{-7*} (2.37–6.31)	97.6 ± 12.6
CP-99994	1.71 × 10 ⁻⁶ (0.26–3.16)	96.1 ± 11.3	9.06 × 10 ⁻⁷ (6.23–12.89)	95.2 ± 10.7

Values are means ± SE, with 95% confidence interval in parentheses. *Significantly different from control, $P < 0.05$.

(Fig. 3, B and D) and the potentiated responses to 10- and 30-Hz EFS stimulation (Fig. 3F) were partly abolished by CP-99994 treatment. EC₅₀ values for ACh and MCh decreased by only 48% and 47% after O₃ exposure in culture in the CP-99994 group compared with 74% and 77% in culture in the saline group (Table 3). EFS-stimulated airway contractions at 10 and 30 Hz increased only by 11% and 8%, respectively, after O₃ in tracheas cultured with CP-99994 compared with 33% and 34% in tracheas cultured with saline. There were no significant effects of CP-99994 on ACh or MCh dose-response curves or EFS-induced contraction (Fig. 3F) in strips from air-exposed animals (Table 3).

Changes in immunoreactive SP-containing intrinsic airway neurons. Because the previous experiments suggested that O₃ exposure might alter SP expression of intrinsic airway neurons, studies were done to examine SP levels in the nerve cell bodies of the ganglia of the LT and the extent of SP innervation in cell bodies of the SMP. About 20% of the LT cell bodies labeled for SP in air-exposed ferrets (Figs. 4A and 5A) and ~50% of the SMP neurons were innervated by SP-containing nerve fibers (Figs. 4C and 5B). After exposure to O₃, >60% of the cell bodies in the LT contained SP (Fig. 4B) and ~90% of the cell bodies in the SMP were innervated by SP-containing nerve fibers (Figs. 4D and 5B).

DISCUSSION

The results obtained from this study show that acute exposure of young ferrets to O₃ produces increased airway responsiveness to cholinergic agonists and enhances smooth muscle contraction during EFS. The elevated airway smooth muscle responses were attenuated by treatment with an NK₁-receptor antagonist before O₃ exposure, indicating that endogenously released SP was involved. A role for tachykinins in causing elevated smooth muscle responses in the airways has been reported, but most previous studies indicate that the source of the tachykinins is sensory neurons innervating the airways (18, 22). Our results are unique, because they suggest that SP levels in intrinsic airway neurons of the LT and innervation density of SMP neurons are increased. Although the involvement of SP released from sensory nerves was not entirely

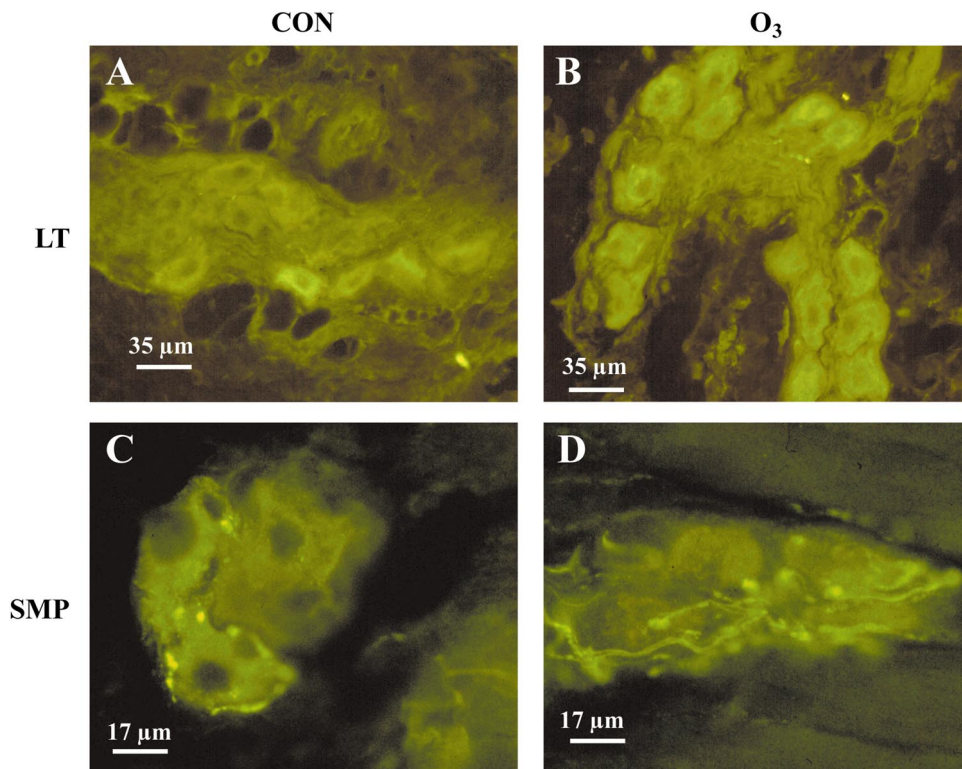
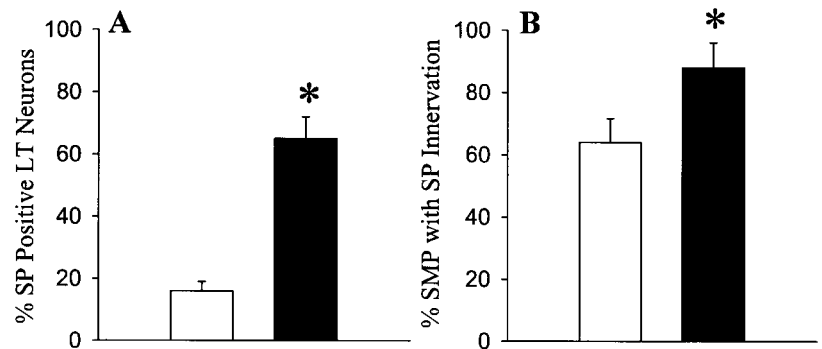


Fig. 4. Fluorescence photomicrographs of substance P (SP)-immunoreactive nerve cell bodies and fibers in longitudinal trunk (LT, A and B) and superficial muscular plexus (SMP, C and D) 1 h after O₃ or air exposure. A: a few SP-immunoreactive LT neurons are seen in the neurons of the control ganglia. Several cell bodies are negative for SP. B: 1 h after O₃ exposure, most of the LT nerve cell bodies contain SP immunoreactivity. C: unlabeled cell bodies in the SMP of control ferret. One cell body is surrounded by a few SP-immunoreactive fibers that form putative synaptic boutons on the cell membrane. D: 1 h after O₃ exposure, several SP-immunoreactive nerve fibers are in close association with the unlabeled SMP nerve cell bodies. Magnification: ×285 (A and B) and ×588 (C and D).

Fig. 5. Effects of exposure to air (open bars) and 2.0 ppm O₃ (solid bars) for 1 h on SP-containing nerve cell bodies in LT (A) and SP innervation of airway neurons in SMP (B). Values are means $\pm$ SE; $n = 6$. *Significant difference between control and O₃-exposed animals, $P < 0.05$.



excluded in this study, the finding that an NK₁-dependent AHR is maintained after 24 h in culture, a procedure shown to cause the loss of SP from airway projections of sensory nerves, suggests that sensory nerves are not the only source for SP in these airways. Our observation that O₃-enhanced airway responses were diminished in tracheal strips cultured with CP-99994 for 24 h suggests that elevated airway responses to O₃ exposure are mediated partly through release of SP from LT neurons and the resulting action of SP either directly on airway smooth muscle or indirectly through modification of SMP neurons.

It should be pointed out that the SP-containing nerve fiber around SMP neurons identified in the present study could have originated from sensory neurons or from LT neurons. Although our studies evaluated airway responsiveness after 24 h in culture, at a time when sensory nerves were probably not present, exposure to O₃ occurred *in vivo* when sensory nerves were still intact. This experimental design does not entirely eliminate the possibility that O₃-induced cholinergic hyperresponsiveness may have resulted from a prolonged effect of SP released by sensory nerve fibers that were viable during the early hours of the culture period. However, SP normally undergoes rapid degradation through the action of neutral endopeptidase in the airways. Therefore, it is expected that the only SP effect after 24 h in culture would result from active release by viable neurons. Because sensory fibers should be substantially degraded by 24 h in culture, the only viable neurons would be neurons in airway ganglia. However, additional studies are needed to determine whether O₃-induced SP released by sensory nerve terminals early in the culture period may produce long-lasting effects, resulting in cholinergic hyperresponsiveness.

The precise neural pathways mediating the enhanced cholinergic responses after O₃ exposure are not clear from the present study. Possible explanations require an evaluation of the intrinsic neural pathways in the ferret airways. Previous anatomic and neurophysiological studies in the airways have identified neural circuits capable of mediating the cholinergic responses after O₃-enhanced SP production in LT neurons. Mitchell and Coburn (25) showed that LT neurons activate individual smooth muscle cells in ferret trachea through cholinergic mechanisms. Our labora-

tory has demonstrated that LT neurons are predominantly cholinergic (5) and that LT neurons project directly to airway smooth muscle (41). These studies suggest that cholinergic pathways from LT neurons to smooth muscle could affect airway smooth muscle directly. One possible way enhanced SP production in LT ganglia could be involved in O₃-induced cholinergic hyperresponsiveness is by modulated cholinergic sensitivity in airway smooth muscle. We hypothesize that O₃ exposure causes enhanced or *de novo* SP synthesis in and release from LT neurons. When LT neurons are activated during or subsequent to O₃ exposure, ACh and SP are coreleased, with SP producing enhanced smooth muscle sensitivity to ACh. Known modulatory effects of SP include the enhancement of cholinergic sensitivity in airway smooth muscle (2, 19, 33, 34, 36).

A second neural pathway potentially involved in O₃-enhanced cholinergic airway smooth muscle responsiveness is the projection from neurons of LT ganglia to neurons of SMP ganglia. Our laboratory showed recently that LT neurons project to and form close, possibly synaptic, connections with VIP- and NO synthase (NOS)-containing cell bodies in SMP ganglia (41). The findings in the present study indicate that O₃ exposure increases SP innervation of these putative VIP/NOS neurons in the SMP, suggesting that SP may modulate the production or release of VIP or NO in SMP neurons. Our laboratory previously showed that >90% of the neurons in SMP ganglia contain VIP and/or NOS. The effect of SP on airway neurons has not been investigated in ferrets. In guinea pigs, however, activation of NK₁ receptors produces enhanced smooth muscle responsiveness mediated through airway ganglia (36). In another study, capsaicin-induced membrane depolarization of guinea pig airway neurons was inhibited by NK₃-receptor antagonists but unaffected by NK₁-receptor antagonists (26). Taken together, these studies suggest that SP release may affect actions of VIP or NO, neurotransmitters associated with the inhibitory nonadrenergic noncholinergic (iNANC) innervation of airway smooth muscle. The exact mechanism remains unknown, since activation of iNANC nerves would seemingly reduce airway responsiveness, and further studies are needed to determine the effects of SP on iNANC neurons. A recent study showed that viral exposure causes a reduction of iNANC innervation with associated enhancement of

airway smooth muscle responsiveness (3), but a possible role of SP in mediating iNANC responses was not investigated.

The potential role of tachykinins such as SP in mediating inflammation, vascular permeability, and smooth muscle contraction in the airway is well known (23). Although SP is generally considered a sensory neuropeptide in the airway and has been associated with neurogenic inflammation mediated through sensory pathways, our laboratory has demonstrated previously that SP is also synthesized in the airway neurons (6, 8). Stimulation of sensory nerve afferents by inhalation of irritants is known to trigger the release of neuropeptides from these afferent endings (22, 24, 37). Pretreatment of animals with a high doses of capsaicin, which is known to cause degeneration of C fibers and deplete SP and NKA in the lung, reduces the magnitude of O₃-induced AHR (18, 19), clearly implicating the involvement of SP in sensory neurons as a mediator of O₃-induced AHR. 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. Our finding that pretreatment with the NK₁-receptor antagonist CP-99994 in vivo partly abolished O₃-enhanced cholinergic and EFS-mediated contractility emphasizes an important role for SP in O₃-induced AHR.

Ollerenshaw and co-workers (29) demonstrated that SP nerve fiber density was increased in airway smooth muscle of patients with severe asthma, and SP and CGRP nerve fiber density was increased in human airway epithelium from subjects with persistent non-productive cough (28). We know of no direct evidence that SP is present in intrinsic airway neurons of humans, although the persistence of SP fibers in lungs from transplant recipients suggests an intrinsic origin for some SP-positive nerves (31). Some studies show that neuronal levels of SP and preprotachykinin mRNA are increased in airway sensory neurons after exposure to toluene diisocyanate (16) or antigen (13). Thus it is clear that airway neurons are able to adapt to a variety of conditions associated with enhanced airway responsiveness by increasing SP production and elevating the levels of SP available for release in the airway wall. The present study clearly demonstrates that O₃ inhalation alters SP levels and innervation of intrinsic airway neurons as well.

In conclusion, the results obtained from this study show that exposure to O₃ induces elevated SP levels in and around airway neurons at the same time that SP-dependent airway smooth muscle responses are increased. Furthermore, pretreatment with the NK₁-receptor antagonist CP-99994 in vivo attenuates O₃-enhanced airway responsiveness to cholinergic stimulation and EFS, suggesting that SP plays an important role in O₃-induced AHR. O₃-enhanced airway responses were also diminished in tracheal strips cultured with CP-99994 for 24 h, indicating that SP may be released from intrinsic airway neurons, although additional studies are needed. Thus these findings show that O₃ exposure enhances SP levels in intrinsic

airway neurons and suggest that neurons in airway ganglia may contribute to O₃-induced AHR.

The authors are grateful to Dr. G. Hobbs (Dept. of Statistics, West Virginia University) for statistical analysis. The authors also thank Pfizer for the supply of CP-99994.

This study was supported by National Heart, Lung, and Blood Institute Grant RO1 HL-35812.

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