

Allergen-induced substance P synthesis in large-diameter sensory neurons innervating the lungs

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Background: Tachykinins such as substance P are localized in unmyelinated slow-conducting C fibers that can be activated by noxious stimuli and tissue inflammation. Substance P is seldom expressed in fast-conducting large-diameter (A-fiber) vagal sensory neurons. We have previously found that allergic inflammation causes a phenotypic change in tachykinergic innervation of the trachea such that the production of substance P is induced in large-diameter sensory neurons projecting mechanosensitive A fibers to the trachea.

Objective: To evaluate whether allergic inflammation also induces substance P synthesis in large-diameter sensory stretch-receptor neurons innervating guinea pig lungs, and to investigate potential mechanisms by which this may occur.

Methods: Sensitized guinea pigs were exposed to allergen (ovalbumin) aerosol. One day later, immunohistochemical analysis was performed on vagal sensory neurons that had been retrogradely labeled from the lungs.

Results: Ovalbumin inhalation caused a significant increase in substance P expression in large-diameter neurofilament-positive nodose ganglion neurons that innervate the lungs ($P < .05$). This effect was decreased by ipsilateral vagotomy.

Exposing isolated nodose ganglia to the sensitizing antigen, ovalbumin, also significantly increased substance P expression compared with control.

Conclusion: Allergic inflammation induces substance P synthesis in large-diameter (A-fiber) nodose ganglion neurons innervating guinea pig lungs. This could contribute to the hyperreflexia seen in allergic airway disease. The full expression of this phenotypic switch in vagus nodose ganglion neurons requires intact vagus nerve, but if allergen reached the systemic circulation in sufficient quantities, it could also affect substance P synthesis by local activation of vagal ganglionic mast cells. (*J Allergy Clin Immunol* 2005;116:325-31.)

Key words: Allergic inflammation, phenotypic switch, neurogenic inflammation, substance P, nodose ganglion, sensory nerve, vagus nerve

In both the somatosensory and visceral-sensory system, sensory neuropeptides, exemplified by the tachykinin substance P (SP), are commonly found stored in the peripheral and central terminals of unmyelinated sensory C fibers.¹⁻³ Inflammatory reactions in the rat paw have been found to evoke a phenotypic switch in the nature of tachykinergic innervation.^{4,5} After a local inflammatory reaction in the paw, the preprotachykinin gene is induced *de novo* in large-diameter, low-threshold, touch-sensitive A β fibers.⁴ Simply touching the paw can then lead to neuropeptide release in the spinal cord that, in theory, could lead to an increase in neurotransmission to the extent that this innocuous touch stimulus is interpreted as a noxious pain-producing stimulus (ie, an allodynia).

A similar inflammation-induced phenotypic switch can occur in vagal sensory innervation of the airways. In the respiratory tract of most mammals, including guinea pigs and human beings, SP-containing fibers are located mainly in the upper airways, trachea, and main bronchi.^{1,6} There are relatively few SP containing fibers in the lungs. In healthy guinea pig trachea and large bronchi, tachykinins are localized nearly exclusively in nociceptive C fibers derived from small-diameter cell bodies in jugular sensory ganglia.^{2,3} One day after allergic inflammation in the lungs, SP production is increased in the lungs and in vagal sensory neurons.⁷ On further analysis, it was determined that part of the increase in SP production occurred *de novo* in large-diameter neurons that project mechanosensitive A δ fibers to the trachea.⁸ This same type of phenotypic switch occurs 2 to 3 days after respiratory tract viral infection.⁹

The touch-sensitive A δ fibers in the guinea pig trachea represent a unique subtype of vagal afferent mechanosensors apparently designed to evoke cough on activation.¹⁰ They differ from classically described intrapulmonary stretch-sensitive fibers in neurophysiology, activation profile, and distribution in the lungs.¹⁰ The intrapulmonary stretch-sensitive A β fibers are further subclassified as either rapidly adapting receptors (RARs) or slowly adapting receptors (SARs). Whether the stretch-sensitive fibers in the lungs can be induced by inflammation to produce neuropeptides in a fashion similar the tracheal touch-sensitive fibers is unknown.

There is also little known about the mechanism by which airway inflammation leads to phenotypic changes

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Abbreviations used

NGF:	Nerve growth factor
RAR:	Rapidly adapting receptor
SAR:	Slowly adapting receptor
SP:	Substance P
Triton-BSA-PBS:	PBS containing 0.3 % TritonX-100 and 1% BSA
Trk:	Tyrosine kinase receptor

of the neurons located in the remote vagal sensory ganglia (nodose and jugular ganglia situated near the base of the brain). A logical scenario is that the allergic reaction leads to the release of certain neurotrophic factors such as nerve growth factor (NGF) that interact with tyrosine kinase receptor (Trk) A on the terminals. Activation of neurotrophin Trk receptors at nerve terminals can signal events to the cell body via internalization and transport of the activated receptors along the nerve axon.¹¹ However, there are alternative mechanisms to consider. The vagal sensory ganglia that contain the cell bodies of vagal sensory neurons are enriched with mast cells. In sensitized animals, antigen can activate these ganglionic mast cells, leading to local mediator release and change in neuronal excitability.¹² It is possible, therefore, that the inhaled allergen is not triggering tachykinin synthesis from within the lungs, but rather, allergen is systemically absorbed and interacts with mast cells locally within the vagal sensory ganglia.

In the current study, experiments were performed to address 2 hypotheses. First, allergic inflammation can lead to induction of tachykinin synthesis not only in tracheal A δ neurons but also in neurons that project A β stretch-sensitive fibers to the lungs. Second, the mechanism for allergen-induced SP production in bronchopulmonary A-fiber nodose neurons requires intact vagal nerve fibers.

METHODS

All experiments were approved by the Johns Hopkins Animal Care and Use Committee. For active sensitization, male Hartley guinea pigs (Hilltop, Scottsdale, Pa) weighing 100 to 300 g were immunized by intraperitoneal injection (10 mg/kg) of ovalbumin (10 mg/mL) dissolved in saline on day 1, day 3, and day 5. On day 21 after the final sensitization, the actively sensitized animals were killed by CO₂ asphyxiation and exsanguinated. The blood was collected, and serum containing ovalbumin-specific IgG₁ was isolated. For passive sensitization to ovalbumin, male Hartley guinea pigs weighing 100 to 300 g were sensitized by intraperitoneal injection of serum (2 mL/kg) containing IgG₁ that was collected from guinea pigs actively sensitized to ovalbumin¹³ and challenged 1 day after sensitization. Control guinea pigs were not injected with serum but challenged in a similar fashion.¹³ The studies evaluating lung-labeled afferent neurons, and the *ex vivo* studies were performed by using actively sensitized animals. The passive sensitization protocol was used in the vagotomy studies as a means to decrease the intra-animal variation in the allergen response. In the *in vivo* studies, sensitized (active or passive) and unsensitized (control) animals were exposed to aerosolized antigen in a Plexiglas chamber (volume, 8 L) with

consecutive increasing concentrations of ovalbumin (0.01%, 0.03%, 0.1% ovalbumin diluted in saline), with 10 minutes of exposure for each concentration to the maximum dose unless the animal developed signs of allergic response (gasping; rapid, shallow breathing; or coughing), whereupon it was removed to breathe ambient air. After 24 hours, the animals were killed with CO₂ inhalation and exsanguinated. We found no difference in the overresponse of the animal to ovalbumin between actively and passively sensitized animals. Nonsensitized animals did not respond to the ovalbumin treatment. The ovalbumin treatment resulted in an eosinophilic bronchitis within 24 hours of exposure (data not shown).

Identification of nonretrogradely labeled large-diameter nodose neurons

We previously reported that allergen challenge induced a phenotypic switch to SP production in large-diameter nodose ganglion neurons (>25 μ m diameter) that project nerve fibers specifically to the trachea.⁸ To evaluate whether allergic inflammation induces a similar phenotypic switch in large-diameter neurons innervating beyond the trachea, we first evaluated all nodose ganglion neurons with diameters larger than 25 μ m for SP immunoreactivity in control animals and after antigen challenge. Nodose ganglia were removed and fixed in 4% formaldehyde (fixative) for 2 hours at 4°C, rinsed 3 times in 0.1 M PBS (pH 7.4) and then cryoprotected with 18% sucrose in PBS for 18 to 24 hours. Serial cryostat sections of the nodose ganglia (12 μ m thickness) were mounted on 4 consecutive slides, such that the first slide had sections 1, 5, 9..., the second 2, 6, 10, and so forth, and the alternate slides were used for the analysis. The sections were dried at room temperature for 30 minutes and rinsed with water and PBS and incubated with blocking solution containing 1% BSA, 10% goat serum, and 0.5% Tween 20 in PBS at room temperature for 1 hour. The slides were then processed for double immunofluorescence staining with a mixture of rabbit polyclonal antibody to SP (1 μ g/mL; Peninsula Laboratories Inc, San Carlos, Calif) and mouse mAb to 160 kd neurofilament protein (13 μ g/mL; Chemicon, Temecula, Calif) diluted in PBS containing 0.3 % TritonX-100 and 1% BSA (Triton-BSA-PBS; Sigma Chemical Co, St Louis, Mo) for 24 hours at 4°C. The sections were washed 3 times with Triton-BSA-PBS and covered with a mixture of goat antirabbit fluorescein (20 μ g/mL diluted in Triton-BSA-PBS; Vector Laboratories, Burlingame, Calif) and goat antimouse Texas red (30 μ g/mL diluted in Triton-BSA-PBS; Vector Lab) for 2 hours at room temperature. The sections were then rinsed twice with PBS and once with higher pH PBS (pH 8.6), then coverslipped with antifade glycerol (Fluoromount, Molecular Probes, Eugene, Ore). Slides were examined under epifluorescence (Olympus DX60 microscope; Olympus Corp, Melville, NY) by using appropriate filter combinations for fluorescein (excitation filter 450-480 nm; barrier filter 500-515 nm) and Texas red (excitation filter 510-550 nm; barrier filter 570-590 nm). The 160-kd neurofilament protein has been reported to be a selective marker for myelinated neurons,^{2,3} most of which are large-diameter neurons (>25 μ m and mean diameter ~40 μ m^{3,8}); only large-diameter, neurofilament-positive, nodose ganglion neurons were evaluated for the expression of SP. Negative controls included slides treated with only secondary antibodies and slides in which available commercially obtained isotype controls were used as the primary antibodies for rabbit and mouse (Vector Lab). These resulted in categorically negative staining.

Identification of lung-projecting large-diameter neurons

Nodose ganglion neurons were retrogradely labeled from the lung with True Blue (Chemicon) a week before sensitization and allergen challenge. True Blue is fluorescent dye that is taken up by the nerve

terminals and travels in membranous vesicles back to the cell soma by retrograde axoplasmic flow. Accordingly, it is extensively used in neurobiology to retrogradely backfill neurons. Guinea pigs were anesthetized with intramuscular injection of ketamine (50 mg/kg) and xylazine (2.5 mg/kg) and received two 1- μ L (3%) transdermal injections of True Blue dye (in PBS containing 1% dimethyl sulfoxide; Molecular Probes) into the right lung using a 5- μ L Hamilton syringe. One week after the retrograde labeling, the animals were exposed to aerosolized antigen as described.

Twenty-four hours after antigen challenge, the animals were killed by intraperitoneal injection of an overdose of sodium pentobarbital (150 mg/kg) and perfused via the ascending aorta with a rinsing solution containing procaine (100 mg/mL) and heparin (10,000 IU/L) in PBS followed by fixative. Intrathoracic organs (lung, trachea, esophagus, heart, and surrounding soft tissues) and nodose ganglia were removed and put into fixative for 2 hours at 4°C, then rinsed 3 times in PBS. Lungs were cut in sections of 1 to 2 mm to identify the dye distribution in parenchyma, blood vessels, and airways. Trachea and esophagus were examined inside and outside the lumens. Ganglia from animals were used only if the dye was delimited primarily to the lung parenchyma and distal airways, with no dye diffusion into the large airways or other extrapulmonary sites.

These nodose ganglia from the allergen challenged animals were prepared and sectioned as described. The alternate slides were examined, and all True Blue-labeled neurons were photographed to identify labeled cells in case some labeled neurons faded after immunostaining. The sections were double stained as described with the exception of using a rat mAb to SP (5 μ g/mL diluted in Triton-BSA-PBS; Chemicon) with the mouse mAb to 160-kd neurofilament (13 μ g/mL diluted in Triton-BSA-PBS; Chemicon) for 24 hours at 4°C as primary antibodies, followed by a mixture of goat antirat antibody labeled with Alexa Fluor 594 (20 μ g/mL diluted in Triton-BSA-PBS; Molecular Probes) and goat antimouse antibody labeled with Alexa Fluor 488 (10 μ g/mL diluted in Triton-BSA-PBS; Molecular Probes) for 2 hours at room temperature as secondary antibodies. The slides were examined as described, except the True Blue was visualized with an UV filter (excitation filter 330-385 nm; barrier filter 400-420 nm). Only True Blue-labeled neurons with neurofilament-positive immunofluorescence were evaluated to determine whether allergic inflammation induces SP production. We tested both SP antibodies in the same ganglion sections and found there was no difference in staining (not shown).

Isolated ganglia studies

The animals were actively sensitized with intraperitoneal injections of ovalbumin as described. Three weeks after the sensitization, the animals were killed by CO₂ inhalation and exsanguinated. Nodose ganglia from both sides were isolated, cut nearly in half to expose the neurons to the antigen *ex vivo*, and placed immediately into Krebs bicarbonate solution (composed of NaCl, 118 mM; KCl, 5.4 mM; NaH₂PO₄, 1.0 mM; MgSO₄, 1.2 mM; CaCl₂, 1.9 mM; NaHCO₃, 25.0 mM; dextrose, 11.1 mM; and gassed with 95% O₂, 5% CO₂, pH 7.4). Each individual nodose ganglion was incubated (37 °C) in 2 mL Krebs bicarbonate solution containing antibiotics (penicillin/streptomycin, 100 U/mL) and continuously oxygenated (95% O₂, 5% CO₂). The solution was changed every 15 minutes 4 times to wash and equilibrate the tissues. The nodose ganglia from the same animal were divided into a control and an antigen challenge group. In the antigen challenge group, ovalbumin (10 μ g/mL) was added to the solution. The ganglia were incubated for various periods (0, 4, 8, 12, 18 hours) at 37 °C and then removed from the test tubes and fixed and processed for double immunofluorescence staining for SP and neurofilament as described in the nonlabeled large-diameter nodose neuron study. We evaluated all large-diameter, neurofilament-positive nodose ganglion

neurons to determine whether antigen induced the expression of SP *ex vivo*.

Vagotomy studies

The animals were anesthetized as described, and 25 μ L 5% Fast Blue dye (Sigma Chemical Co, St Louis, Mo) was instilled into the trachea as describe previously.¹⁴ A week later, the animals were divided into unsensitized (control) and sensitized groups. In the sensitized group, the animals were passively sensitized with intraperitoneal injection of ovalbumin-sensitized serum as described. A day after the sensitization, under general anesthesia as described, the vagus nerve was unilaterally ligated caudal to the nodose ganglion with suture in 2 places approximately 1 cm apart, and the section of vagus nerve between sutures was removed. On the following day, the animals were challenged with ovalbumin inhalation as described. Unsensitized (control) animals had a similar unilateral vagotomy and were challenged with ovalbumin. Twenty-four hours after the antigen challenge, the animals were killed with CO₂ inhalation and exsanguinated. The nodose ganglia were removed bilaterally and fixed for double immunofluorescence staining with antibodies to neurofilament (160 kd) and SP as described in the nonlabeled large-diameter nodose neuron study. Only Fast Blue-labeled neurons that were neurofilament-positive were evaluated. For vagotomy, Fast Blue was used; no differences in labeling were noted between Fast Blue and True Blue, and the latter was used for the lung labeling procedure described.

Statistical analysis

The data were analyzed by using the Student paired or nonpaired *t* tests. Data were expressed as means $\pm$ SEMs. *P* < .05 was considered significant.

RESULTS

Nonretrogradely labeled large-diameter nodose neuron study

We have previously made exhaustive studies of cell diameters and neurofilament staining in vagal sensory neurons projecting to the respiratory tract.^{3,9} The cell diameters of neurofilament-negative neurons form a unimodal population with a mean diameter of about 20 μ m. The cell diameters of neurofilament-positive neurons also form a unimodal distribution with an average diameter of 43 ± 8 (n = 713) in one study and 40 ± 3 (n = 307) in another study. In the current study, we evaluated 781 nodose ganglion neurons that were <25 μ m in diameter and found that >99% were neurofilament-negative, whereas among the 792 neurons with diameters >30 μ m, all were neurofilament-positive. On the basis of these studies, and in accordance with conclusions drawn from more direct studies in the somatosensory system,¹⁵ we conclude that the large diameter neurofilament-positive population of neurons project myelinated A fibers to peripheral tissues.

In unsensitized (control) animals, we found that only 78 of 2034 large neurons were SP-immunoreactive (average of $3.8\% \pm 0.6\%$; n = 4 animals). In ovalbumin-sensitized animals, the number of large-diameter neurons positive for SP was substantially higher, 712 of 1939 (average of $36.7\% \pm 3.1\%$; n = 4 animals; *P* < .01; compared with unsensitized animals, Fig 1, C; part of these data was

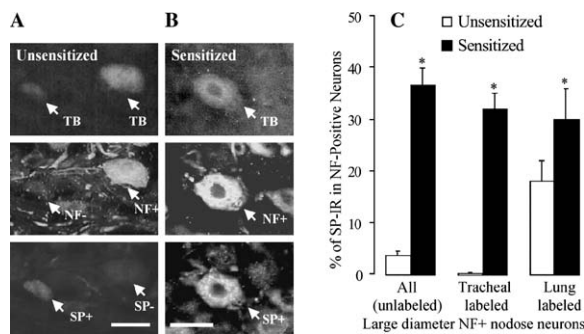


FIG 1. SP immunoreactivity (SP-IR) in nodose ganglion neurons 1 day after the antigen challenge with ovalbumin (OVA) inhalation in (A) unsensitized (control) and (B) sensitized guinea pigs. Representative photomicrographs of lung neurons labeled with True Blue (TB) dye with double immunofluorescence staining of SP and neurofilament (NF; 160-kD) antibodies (scale bar = 50 μ m). C, Histogram showing the percentage of SP immunoreactivity in large-diameter NF-positive neurons in nodose ganglia after OVA challenge in unlabeled (n = 4), tracheal-labeled (n = 3), and lung-labeled (n = 8, unsensitized; n = 9, sensitized). Note, tracheal labeled neuron data taken from our previous study.⁸ Data are presented as the means $\pm$ SEMs. * P < .01 between sensitized and unsensitized animals).

obtained from our previous study⁸ and is included here for comparison purposes).

Lung-labeling experiment

To address more specifically the hypothesis that SP synthesis could be induced in intrapulmonary A-fiber neurons, we used a retrograde labeling strategy. The retrograde tracer True Blue was injected into right lung. One week after the injection, both actively sensitized and unsensitized (control) groups were challenged with inhaled ovalbumin. The animals were killed 1 day after antigen challenge, and the distribution of the dye was examined in the thoracic tissues. Thirty-two animals were used for this experiment. In 17 animals, we confirmed that the dye was delimited primarily to the lung parenchyma and distal airways, with no dye diffusion into the large airways or extrapulmonary sites. Fifteen animals were excluded from further analysis as a result of diffusion of the dye to the large airways and/or cardiac tissue.

Among the successfully labeled animals, 9 animals were unsensitized (controls) and 8 were sensitized. All animals were challenged with ovalbumin, and 1 day later, the neurons were evaluated for SP immunoreactivity. In control animals, an average of $18\% \pm 4\%$ of the large-diameter neurofilament-positive neurons were SP-immunoreactive. In sensitized animals challenged with ovalbumin, $30\% \pm 6\%$ of large-diameter neurofilament-positive neurons were SP-positive (P < .05 compared with control animals; Fig 1, C).

Isolated ganglia studies

We have previously noted that mast cells located within the nodose ganglia isolated from ovalbumin-sensitized

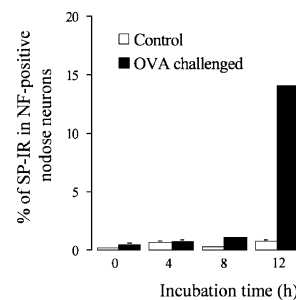


FIG 2. Nodose ganglia (*ex vivo*) were treated ovalbumin (OVA; 10 μ g/mL) or saline for the times indicated (n = 1-2 for 0, 4, 8, 12 h and n = 8 for 18 h). At 18 hours after OVA treatment, the percentage of SP immunoreactivity (SP-IR) is significantly increased in large-diameter (>25 μ m) neurofilament (NF)-positive neurons ($16\% \pm 2\%$; n = 8) compared with the control ($2\% \pm 0.8\%$; n = 6). Data are presented as means $\pm$ SEMs. * P < .01.

animals degranulate and release inflammatory mediators on antigen challenge.¹² We therefore next addressed the question whether a local allergic reaction within the nodose ganglion is sufficient to induce SP expression in large-diameter neurofilament-positive neurons.

The nodose ganglia were isolated from actively sensitized animals and exposed to 10 μ g/mL ovalbumin or vehicle (Krebs bicarbonate solution) *ex vivo*. Ovalbumin effectively induced SP synthesis in neurofilament-positive neurons beginning at between 8 and 12 hours of exposure. After 18 hours of ovalbumin treatment, approximately 16 % of neurofilament-positive neurons were SP-positive (n = 8), compared with $\sim 2\%$ in the vehicle treated ganglia (n = 6; P < .01; Fig 2).

Vagotomy studies

The data from the isolated ganglia studies suggest that if inhaled ovalbumin can reach the vagal sensory ganglia in sensitized animals, it may be capable of acting locally to induce SP synthesis in neurofilament-positive neurons. To address this question further, *in vivo* experiments were conducted in passively sensitized (or control) animals with unilateral vagotomy performed before ovalbumin inhalation. The induction of SP immunoreactivity in the nodose ganglion neurons was compared between the vagotomy side and contralateral side with intact vagus. If the allergen-inducing signal for SP synthesis travels to the cell body via the nerve fiber, then the induction will be noted only in the ganglia associated with an intact vagus nerve. In these studies, either the right or left vagus nerve was severed in a given animal. The data obtained were the same whether the right or left ganglia was studied, and therefore, the results were pooled.

Fast Blue dye (25 μ L) was instilled into cervical trachea 1 week before the antigen challenge. This procedure labeled both extrapulmonary and intrapulmonary compartments. All animals were then challenged with aerosolized ovalbumin. Twenty-four hours after the antigen challenge, the animals were killed, and labeled neurofilament-positive neurons were evaluated for the SP immunoreactivity.

Vagus nerve intact side. In unsensitized animals, $3\% \pm 1\%$ ($n = 4$) of neurofilament-positive neurons expressed SP in nodose ganglia of an intact vagus nerve. As expected, in sensitized animals, the percentage of neurofilament-positive neurons expressing SP in nodose ganglia with intact vagus averaged $29\% \pm 3\%$ ($n = 4$; Fig 3).

Vagus nerve severed side. In unsensitized animals, $9\% \pm 1\%$ of the neurofilament-positive neurons were SP-positive in nodose ganglia on the side in which the vagus nerve was cut. Comparing these results with those observed in ganglia obtained from the side with an intact vagus ($3\% \pm 1\%$) suggests that severing the vagus *per se* signals the induction of tachykinin synthesis in a subset of neurofilament-positive nodose neurons. In sensitized and challenged animals, there was a significant ($P < .05$) but slight increase in the percentage of the neurofilament-positive neurons that were SP-positive in nodose ganglia on the side of the vagotomy (Fig 3). However, this apparent ovalbumin effect in ganglia ipsilateral to the vagotomy (1.8-fold increase) was significantly less than that observed on the vagus intact side (9.7-fold increase).

DISCUSSION

The results support the hypothesis that allergic inflammation in guinea pig lungs can lead to a switch in sensory SP innervation such that, in addition to sensory C fibers, SP is found in cough-causing A fibers in the trachea⁸ and in stretch-sensitive A fibers in the lungs (current study). In addition, the results are consistent with the hypothesis that this phenotypic switch in tachykinergic innervation requires signals that, at least in part, reach the cell bodies in the distant sensory ganglia via the vagal nerve fibers.

In healthy mammals, most neuronal tachykinins (SP and neurokinin A) in the mammalian respiratory tract are found in sensory C fibers.^{1,2} The vagal C-fiber neurons in the guinea pig respiratory tract are derived from cell bodies situated in the jugular and nodose ganglia.^{2,3,16} The jugular C fibers, found in extrapulmonary airways and intrapulmonary bronchi, are more likely to contain SP than the nodose C fibers located within the lungs.¹⁶ Regardless of the type of C fiber, these nerves are typically not thought to be activated in healthy animals under normal circumstances. Rather, they are recruited to action by noxious stimuli or pathological conditions such as tissue inflammation.¹⁷ On activation of a tachykinin-containing nerve, the tachykinins are released into synapses in the central nervous system, where they serve to evoke pain in the somatosensory system^{4,18} and augment cough^{19,20} and parasympathetic reflexes in the respiratory system.²¹⁻²⁴ When released from the peripheral nerve terminals via axon reflexes, the tachykinins may cause or contribute to local inflammatory reactions.²⁵⁻²⁷

Substance P is seldom expressed in large-diameter (A-fiber) sensory neurons innervating the trachea or lungs.^{2,3,28} The large-diameter neurons innervating the guinea pig respiratory system are located mainly in the nodose ganglia and project touch-sensitive and stretch-

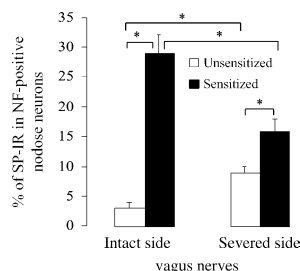


FIG 3. Histogram demonstrating the percentage of SP immunoreactivity (SP-IR) in Fast Blue-labeled neurofilament (NF)-positive nodose neurons in intact versus severed vagus nerves 1 day after the ovalbumin inhalation. Data are presented as means $\pm$ SEMs; $n = 4$ per group. * $P \leq .05$.

sensitive A fibers to the trachea and lungs, respectively.^{3,10,16} Fischer et al⁷ were first to publish that allergic inflammation in guinea pig airways is associated with an increase in preprotachykinin gene transcription and SP synthesis in nodose ganglion neurons. Subsequently, we showed that this could occur in large-diameter neurofilament-positive neurons that projected low-threshold touch-sensitive A δ fibers to the trachea.⁸ Further investigation indicated that tracheal nodose A δ fibers in the guinea pig represent a unique type of sensory nerve that evokes cough on activation.¹⁰

In the current study, we designed experiments to determine the effects of allergic inflammation in the lung on SP content of neurons that project A fibers to intrapulmonary structures (ie, RAR/SAR population). We injected small volumes of dye directly into the lung parenchyma and subsequently evaluated retrogradely labeled neurofilament-positive neurons. In the allergen-challenged animals, about 30% of the lung-specific neurofilament-positive neurons expressed SP immunoreactivity. This value is similar to that observed in tracheal-labeled neurons. We have previously reported that under control conditions, very few (1% to 3%) of neurofilament-positive neurons in the nodose ganglia express SP immunoreactivity.⁸ Likewise, Kummer et al² have reported that nodose neurons labeled from healthy guinea pig lungs are essentially all SP-negative. We were therefore surprised by our finding that 18% of the neurofilament-positive neurons labeled from the lung were SP-positive in the control animals. We suspect that this may be a result of some inflammation caused by our intraparenchymal injections of the dye itself. In any event, the results support the hypothesis that allergic inflammation can lead to induction of tachykinin synthesis in stretch-sensitive A fibers within the lungs.

In our previous electrophysiological analysis of nodose A fibers projecting to the guinea pig lungs, we found that they represented a rather homogenous population of fast-conducting stretch-sensitive nerves.¹⁶ All of the fibers had conduction velocities in the range of 9 to 25 m/s and could be segregated on the basis of their adaptation to prolonged lung inflation into either RAR or SAR phenotypes. The finding that 30% of nodose A-fiber neurons express SP after allergen challenge indicates that SP production can

be induced in some vagal stretch-sensitive nerves. This raises the possibility that SP could be released from their central terminals in the brain stem during breathing, independently of noxious stimuli. This would be analogous to the phenotypic switch described in touch-sensitive A β fibers of the somatosensory system, in which such an effect has been suggested to underlie sensations of allodynia.⁴

How allergic inflammation causes increased SP immunoreactivity in nodose neurons is not known. By using a similar model system, Fischer et al⁷ provided direct evidence that allergen-induced increase in tachykinin synthesis occurs at the level of preprotachykinin gene transcription in the cell nucleus. We considered 2 pathways by which inhaled allergen can influence gene transcription in the cell bodies located at a distant vagal sensory ganglion. First, the allergen could reach the systemic circulation and activate mast cells within the ganglion, causing a local effect on the neighboring neurons. Second, the allergic inflammation within the airway wall could influence the nerve terminals in a manner that sends long-distance signals to the cell nucleus via the afferent nerve fiber.

When the nodose ganglia isolated from sensitized guinea pig are subsequently exposed to the sensitizing antigen, ganglionic mast cells degranulate, mast cell-associated mediators are released, and the electrical excitability of resident neurons is altered.¹² In the current study, we found that antigen administered to the nodose ganglia *ex vivo* caused a ~10-fold increase in the percentage of SP-expressing neurofilament-positive neurons. This effect is presumably secondary to the activation of resident mast cells, although this hypothesis was not further addressed. On the basis of these findings, it can be surmised that if inhaled ovalbumin reached the systemic circulation in sufficient quantities, it could affect SP synthesis in nodose neurons via a local activation of ganglionic mast cells.

The experiment in which the vagus nerve was unilaterally severed 1 day before the allergen inhalation more directly addresses this issue. Regrettably, severing the nerve itself appeared to increase the SP production in neurofilament-positive neurons to nearly 10% (compared with 3% with the vagus nerve intact). This is consistent with the findings of Noguchi et al,^{29,30} who reported that severing the peripheral fibers *in vivo* in the somatosensory system leads to the induction of neuropeptides production in dorsal root ganglion neurons. This vagotomy-induced increase in SP immunoreactivity in neurons clouds the interpretation of the data. Nevertheless, allergen inhalation caused the expected robust increase (9.7-fold) in the percentage of neurofilament-positive, SP-positive nodose neurons only in the side with the vagus nerve intact. On the side in which the vagus was severed, ovalbumin inhalation was associated with only a marginal increase (1.8-fold) in the percentage of neurofilament-positive neurons expressing SP. This is the expected result if the site of action of the allergen is within the lung, but it would not be expected if the allergen were acting at the level of the sensory ganglia.

Also supporting the hypothesis that the site of initiation of the phenotypic switch is within the lung is the finding that the effect of allergic inflammation on SP induction in large-diameter neurons can be mimicked with respiratory tract viral infection.⁹ The virus infection in this model is thought not to extend beyond the airway epithelium. The small ovalbumin-induced effect observed in nodose neurons ipsilateral to the vagotomy may have been a result of the fact that the vagus was cut caudal to the point where the superior laryngeal nerve meets the vagus. Thus, the pathway remained intact for that population of A fibers that project to the larynx and pharynx via the superior laryngeal nerve.

The question remains as to the nature of the chemical mediators capable of being released after allergic inflammation that can lead to long-distance nuclear signaling via the vagal axons. On the basis of the literature, it seems likely that a neurotrophin molecule such as NGF may be causally involved.³¹ Neurotrophins and their Trk receptors are, in fact, designed to signal gene transcriptional events from interactions occurring at distant terminals.¹¹ In addition, NGF is associated with allergic reactions,³² and we have previously reported that microinjection of NGF into the tracheal wall leads to SP production in large-diameter neurofilament-positive nodose neurons innervating the trachea.³³ Similar results have also been observed in the mouse.³⁴

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