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EFFECTS OF SILICA EXPOSURE ON SUBSTANCE P IMMUNOREACTIVITY AND PREPROTACHYKININ mRNA EXPRESSION IN TRIGEMINAL SENSORY NEURONS IN FISCHER 344 RATS

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Trigeminal sensory neurons innervate the nasal cavity and may release substance P (SP) upon exposure to inhaled irritants. The purpose of this study was to determine if silica dust, an occupational irritant causing inflammation, activates sensory neurons supplying the nasal cavity. Male Fischer 344 rats were placed in inhalation chambers and exposed daily to 2 mg/m^3 of fresh silica (average diameter 1 μm) for 6 mo. Following exposure, the trigeminal ganglia (TG) were removed and prepared for SP immunocytochemistry and for preprotachykinin (PPT) autoradiographic in situ hybridization. The SP-like immunofluorescence in TG neurons was subjectively categorized as high, moderate, or low (background) intensity. In situ hybridization autoradiographs were quantified on the basis of grain density using digital imaging analysis. The SP immunoreactivity and PPT mRNA expression in the TG neurons were significantly increased after silica inhalation. The proportion of highly positive SP-immunoreactive neurons shifted from 1.30 ± 0.58% in controls to 11.30 ± 1.15% after silica treatment. The neurons exhibiting high grain density for PPT mRNA increased from 1.50 ± 0.87% in controls to 11.67 ± 0.58% in the silica group. Thus, inhalation of silica causes upper airway irritation resulting in increased levels of immunoreactive neuronal SP and PPT mRNA. These findings suggest that silica activates sensory pathways that may be involved in nasal inflammation.

Silica is a common contaminant in dusts generated in a variety of occupational settings including mining, sand blasting, and stone cutting (Wagner, 1997). The inhalation of coal mine dust has been well documented as the cause of classical pneumoconiosis in which the alveolar walls become inflamed and eventually fibrotic (Attfield & Seixas, 1995;

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Maclaren & Soutar, 1985). Chronic silicosis can develop over a period of 20–40 yr and may progress from simple silicosis with few symptoms to progressive massive fibrosis where restrictive lung impairment is evident. Prolonged inhalation of dust containing silica is associated with symptoms of obstructive airway disease including reduced ventilatory capacity, chronic phlegm production, chronic bronchitis, breathlessness, and wheezing (Lapp et al., 1972; Kibelstis et al., 1973; Chia et al., 1992; Seixas et al., 1992). Cellular injury and tissue damage are believed to be main factors responsible for the development of silicosis (Rom et al., 1987). The inhalation of dust also affects the upper respiratory tract as in rhinitis and nasal irritation (Chan-Yeung et al., 1978). Previous studies have demonstrated that upper airway irritation by cigarette smoke, toluene diisocyanate, formalin, histamine, and sulfur dioxide activates nerve fibers in the airway mucosa, which can induce neurogenic inflammatory responses (Lundberg et al., 1987). These responses include mucous secretion, vasodilation, plasma extravasation, vascular permeability, and bronchconstriction. Despite clinical observations, there are few if any reports concerning the association between pathological developments and possible neural and inflammatory mechanisms in the upper nasal airways due to chronic silica inhalation.

Airway responses due to irritant inhalation result from pulmonary reflexes mediated by sensory and autonomic nerve fibers in the airways (Barnes, 1986). Nerve fibers in the airways and the mediators they release may be involved in regulating normal as well as abnormal responses to inhaled dusts. In the upper airways, the nasal mucosa is provided with sensory innervation from the trigeminal ganglia (Grunditz et al., 1994). One mediator implicated in the sensory regulation of airway responses is substance P (Baraniuk et al., 1991; Gawin et al., 1993). Substance P (SP) is found localized within cell bodies of the trigeminal ganglia and in the peripheral terminals of sensory nerves located in arteries, veins, mucous glands, and epithelium (Lee et al., 1985; Alvarez et al., 1991; Reuss et al., 1992). The nerve fibers projecting to the nasal mucosa are extensively branched, small-diameter, unmyelinated C-fibers that convey painful sensation after mechanical, thermal or chemical stimulation (Lundblad et al., 1983; Lundblad, 1984). The induction of neurogenic inflammation is believed to be induced by the release of SP from capsaicin sensitive C-fibers in the airway mucosa (Jancso et al., 1967). Stimulation of sensory nerves innervating the vascular beds of the nasal mucosa causes an increased release of SP, resulting in vasodilation (Widdicombe, 1990). Furthermore, the increased SP levels and neurogenic inflammation in the nasal mucosa after a nasal allergen provocation with pollen are reduced after SP antibodies (Nieber et al., 1991).

The purpose of this investigation was to determine if chronic inhalation of silica would activate sensory neurons supplying the nasal cavity. Therefore, substance P immunoreactivity in trigeminal sensory neurons

was monitored. Since an increase in SP release may deplete nerve terminals of neuropeptides, it was hypothesized that neuropeptide synthesis may also increase. The preprotachykinin-I (PPT-I) gene can generate three different mRNAs, whereas the α-PPT mRNA encodes specifically for SP alone. Therefore, in this study, preprotachykinin mRNA expression in trigeminal neurons was examined as a possible mechanism responsible for maintaining intraneuronal SP levels in response to inhaled irritants.

MATERIALS AND METHODS

Six Fischer 344 male rats, 175–200 g, were used for inhalation exposure studies to determine if silica dust activates sensory neurons in the trigeminal ganglia that innervate the nasal cavity. The experimental design consisted of two groups: a control group of three rats that inhaled ambient or filtered air, and a experimental group of three rats that inhaled silica dust. Male Fischer 344 rats were placed in inhalation chambers and exposed daily (8 h/d) to 2 mg/m^3 of fresh silica (diameter average 1 μm) or filtered air for 6 mo. After the inhalation exposure period, the experimental and control animals were given a lethal injection of sodium pentobarbital (90 mg/kg intraperitoneal; Nembutal, Abbott Laboratories, Chicago). The right and left trigeminal ganglia were dissected out and prepared for SP immunocytochemistry and preprotachykinin (PPT) in situ hybridization, respectively.

Silica Inhalation Exposure

For the inhalation exposure, rats were divided into two groups and placed in Hazelton 2000 exposure chambers. Food and water were available ad libitum. Rats in one chamber were exposed for 8 h/d, 5 d/wk to filtered air containing 2 mg/m^3 silica (the maximum allowable coal mine dust concentration). Silica for the exposures (IOTA standard quartz 955, between 75 and 300 μm, Unimin Corporation) was fractured in a Jet-O-Mizer mill (Fluid Energy Processing and Equipment Co.) to an average size of 1 μm. The aerosolized fresh dust was passed through a TSI model 3054 aerosol neutralizer before being injected into the Hazelton exposure chamber (air is ducted from the outside and passed through a HEPA filter to remove particulates). Dust concentration in the chamber was monitored by a RAM-1 real-time dust monitor. The other chamber received filtered air only. Rats were removed after 6 mo.

SP Immunocytochemistry

Tissue Preparation The control and experimental animals were euthanized by an anesthetic overdose using sodium pentobarbital. The right trigeminal ganglia (TG) were dissected out by cutting distal to the division of the ophthalmic, maxillary, and mandibular divisions and at the junction of the trigeminal nerve (V) emerging with the ganglion. The

ganglia were fixed in picric acid–formaldehyde (PAF) fixative for 3 h (Stefanini et al., 1967) and rinsed 3 times with 0.1 *M* phosphate-buffered saline (pH = 7.8) containing 0.3% Triton X-100 (PBS-Tx). During the final rinse, the tissues remained in the PBS-Tx overnight.

For cryostat sectioning, the ganglia were oriented on corks so the first section taken would be from the ventral surface. Then the ganglia were frozen in isopentane, cooled with liquid nitrogen, and stored in airtight bags at –60°C.

Every fifth section (12 μm thickness) of the trigeminal ganglia was collected on subbed coverslips. The first coverslip was utilized for immunocytochemistry to determine the SP-containing neuronal cell bodies in the trigeminal ganglia.

Immunocytochemical Procedures Immunocytochemical procedures for localizing SP-immunoreactive neurons are identical to those described previously (Dey et al., 1990). Briefly, cryostat sections on coated coverslips were covered with rabbit anti-SP antiserum (Peninsula, Belmont, CA) diluted 1:100. The coverslips were incubated in a humid chamber at 37°C for 30 min. The coverslips were rinsed with a 1% bovine serum albumin–phosphate saline buffer (pH = 7.8) containing Triton X solution (PBS-Tx + BSA) 3 times, allowing 5 min for each rinse. The sections on the coverslips were covered with fluorescein isothiocyanate-labeled goat anti-rabbit immunoglobulin G (IgG; ICN Immunobiologicals) diluted 1:100. The sections were then incubated at 37°C for 30 min. The coverslips were rinsed again in PBS-Tx + BSA 3 times for 5 min. The coverslips were mounted with fluoromount (Southern Biotechnology, Birmingham, AL) and observed with an Olympus BH-2 fluorescence microscope equipped with a fluorescein filter (excitation wavelengths from 455 to 500 nm and emission wavelengths >500 nm).

Controls for specificity of primary antiserum consisted of absorption of 1 mg/ml antiserum with SP. Nonspecific background labeling was determined by omission of primary antiserum. Even after these controls were employed, however, cross-reactivity of antiserum with other known or unknown peptides present in the tissue cannot be excluded by immunocytochemical procedures. Therefore, the terms SP immunoreactivity (SP-IR) or SP-like immunoreactivity (SP-LI) are used here when referring to labeled structures.

Qualitative Analysis of SP Immunofluorescence SP immunoreactivity of the neuronal cell bodies in the trigeminal ganglia was evaluated by direct observation. The fluorescence intensity of the SP-IR neurons was categorized as high, moderate, or low. Category determinations were done by an observer without prior knowledge of the experimental groupings. The results were analyzed using parametric analysis of variance (ANOVA) statistical analysis. The percent of SP-IR neurons in each category was calculated by dividing the number of cell bodies in the high, moderate, or low categories by the total number of neurons in the ganglion.

Preprotachykinin In Situ Hybridization Tissue Preparation

Fresh, unfixed left trigeminal ganglia were excised, frozen on cork, and sectioned as described already. The sections were placed on gelatin-chromalum-coated slides and gently dried using a hair dryer. Continuous serial sections were collected on six slides. The first two slides were used for preprotachykinin anti-sense (PPT-AS) probe, the second two slides were used for PPT-AS + Rnase, and the final two slides were utilized for PPT sense (PPT-S) probe.

Probe Generation The rat SP cRNA probes used in this study were generated from a plasmid containing a rat β-preprotachykinin cDNA (Brown et al., 1990). The plasmid pGlβ-PPT was linearized by using the restriction enzyme Hind III. Next a radiolabeled probe complementary to the coding sequence of rat β-PPT (PPT-AS probe) was transcribed from the plasmid template by using ^{35}S-UTP (≥1000 Ci/mmol; Amersham Life Sciences, Arlington Heights, IL) and T_7 polymerase (Promega, Madison, WI). A cRNA sense probe was also synthesized following linearization with Eco-RI and transcription with SP-G polymerase.

In Situ Hybridization Procedure In situ hybridization procedures for localizing PPT mRNA are similar to those described previously (Kiyama & Emson, 1990). Briefly, the slides were removed from the dessicator and incubated for 30 min at room temperature in 4% paraformaldehyde. Next the slides were rinsed in PBS/diethylpyrocarbonate (DEPC) and pretreated with a mixture of 1.5% triethanolamine, 0.25 acetic anhydride, and 0.9% NaCl. The slides were dehydrated with ethanol and then delipidated with chloroform. For negative controls, a few slides were treated with RNase A (100 mg/ml) and RNase T_1 (5 mg/ml) in Tris ethylenediamine tetraacetic acid (EDTA) saline (TES) buffer (pH = 7.5) for 30 min at 37°C. All other slides were pretreated only with TES buffer at 37°C. After RNase treatment, all slides were rinsed in TES at room temperature, and incubated in 4× saline sodium citrate (SSC) twice for 10 min at room temperature. The slides were placed in humidified chambers and the sections were incubated with 300 ml of hybridization solution at 37°C overnight. The hybridization mixture contained 4× SSC, 50% deionized formamide, 1× Denhardt's solution, 500 mg/ml sheared salmon sperm DNA, 10% dextran sulfate, 10 m*M* dithiothreitol, and the ^{35}S-radiolabeled β-PPT probes. The slides were removed from the chambers, rinsed in 2× SSC, and incubated in an RNase A (100 mg/ml)–SSC solution for 30 min at 37°C. Finally, the slides were rinsed in a 2× SSC solution, dehydrated, and dried completely. The autoradiography was performed by dipping each slide in Kodak NTB2 nuclear emulsion in a 1:1 ratio with water at 43°C. The slides were stored in a light-tight chamber and exposed in the dark at 4°C for 10 d. Slides were removed from the boxes, processed using Kodak D19 developer, and counterstained with hematoxylin and eosin.

Quantitative Analysis of Autoradiographs Image analysis was used to quantify β-PPT mRNA expression in the neuronal cell bodies of the

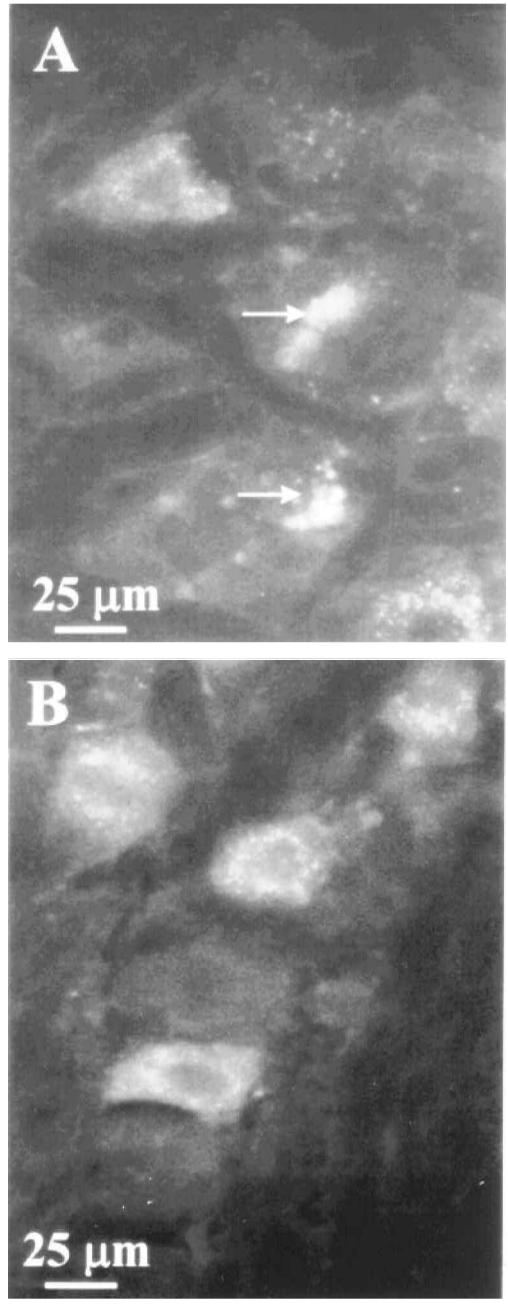

FIGURE 1. Photomicrographs showing the localization of SP immunoreactivity in trigeminal neurons from (A) control and (B) silica-treated rats (arrows indicate autofluorescence present in some neurons).

trigeminal ganglia. The sections were examined under light field with a Zeiss Axiovert Microscope at 20× magnification and a Dage MT 1 CCD72 camera (Michigan City, ID). Autoradiographic density was determined for every nerve cell body in the trigeminal ganglia sections. The grain density was quantitated by Oncor image analysis system (Rockville, MA). The individual trigeminal neurons were encircled and the grain density per area of neuron was computed. The overall percent of grain density was calculated by dividing the total cell body area by the grain density area. The percent of grain density was categorized as high (66.8–100%), moderate (33.4–66.7%), or low (0–33.3%).

Statistical Analysis

After the mean number of neurons in each category were calculated, the three groups were compared by one-way ANOVA using the Macintosh-based software Statview II. Significance was set at $p \leq .05$. Upon obtaining the significant F values, the individual means were compared using the least significant difference test.

The mean values of each SP mRNA grain density were calculated for all three categories and the same statistical comparisons were used as described previously.

RESULTS

Silica-Induced Changes in SP-Like Immunofluorescence in Trigeminal Neurons

SP immunoreactivity was significantly increased in trigeminal neurons after a 6-mo silica exposure. Silica inhalation produced an increased in the number of SP-containing neurons with high and moderate fluorescent intensity in comparison to the control (Figures 1A, 1B, and 2). The proportion of highly positive SP-immunoreactive neurons markedly shifted

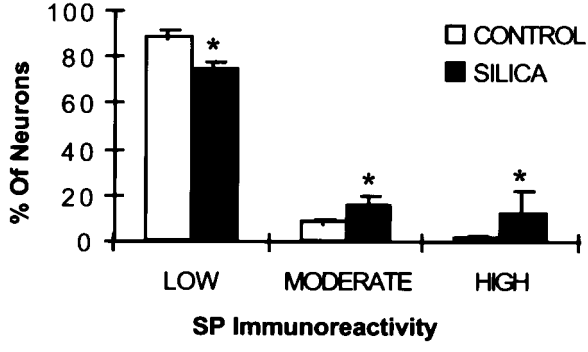

FIGURE 2. The percent of neurons demonstrating low, moderate, or high SP immunofluorescence from rats exposed to silica daily for 6 mo compared to control. Control rats received no silica exposure. Asterisk indicates significantly different from control, $p \leq .05$ (silica $n = 3$, control $n = 3$).

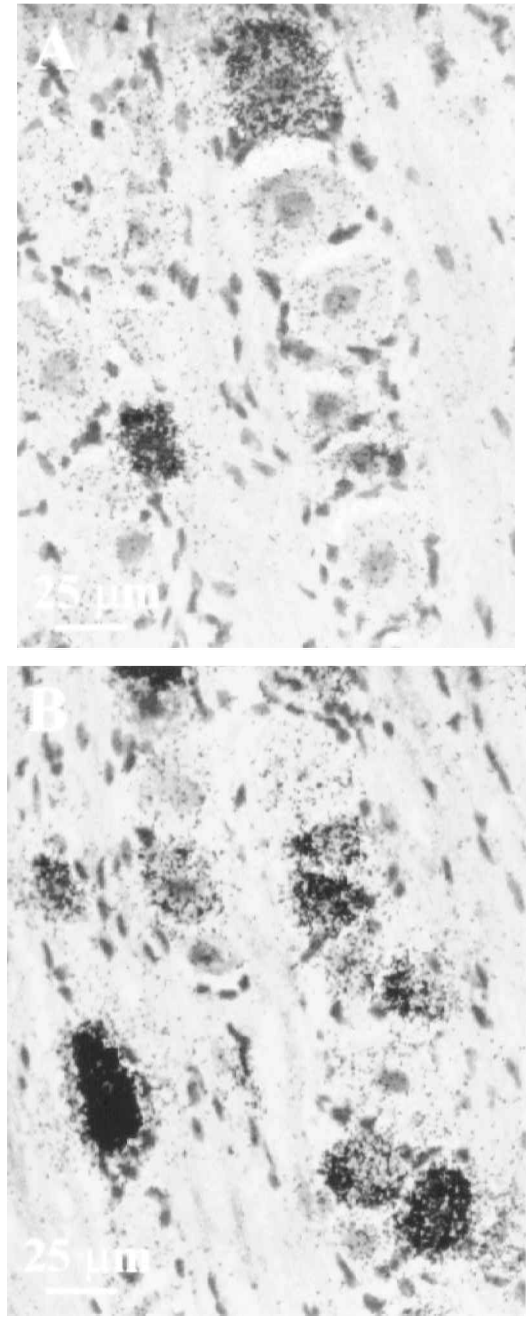

FIGURE 3. Photomicrographs showing the localization of preprotachykinin mRNA in trigeminal neurons from (A) control and (B) silica-treated rats.

from 1.30 ± 0.58% in controls to 11.30 ± 1.15% after silica inhalation. The percent of moderate SP-immunoreactive neurons significantly increased from 8.33 ± 1.52% in controls to 15.33 ± 4.04% when silica treated. There was a significant decrease in the percent of SP-containing neurons with low fluorescent intensity in comparison to the control. The low SP immunoreactivity in trigeminal neurons decreased from 88.33 ± 3.79% in the controls to 74.00 ± 4.36%.

Silica-Induced Changes in SP mRNA in Neurons of the Trigeminal Ganglia

The PPT mRNA expression in the trigeminal neurons was significantly increased after a 6 month silica inhalation (Figures 3A, 3B, and 4). Silica inhalation produced a significant increase in the number of SP-containing neurons with high and moderate grain density in comparison to the control. The neurons exhibiting high grain density increased from 1.50 ± 0.87% in controls to 11.67 ± 0.58% in the silica group. The proportion of neurons demonstrating moderate grain density shifted from 12.16 ± 2.14% in controls to 20.44 ± 8.05% after silica treatment. After silica exposure, a significant decrease in the percent of SP-containing trigeminal neurons with low grain density was produced in comparison to control. The low grain density decreased in neurons from 88.2 ± 2.43% in control to 65.1 ± 1.53% in the silica group.

DISCUSSION

This study demonstrates that a 6-mo inhalation of 2 mg/m^3 silica dust results in significantly increased levels of immunoreactive SP and PPT-mRNA in sensory neurons of the trigeminal ganglia. These findings rep-

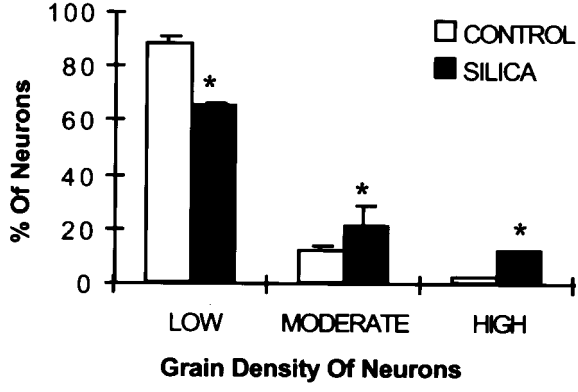

FIGURE 4. Percent of neurons demonstrating low, moderate, or high SP grain density (mRNA) from control or silica exposed rats. Asterisk indicates significantly different from control, $p \leq .05$ (silica $n = 3$, control $n = 3$).

resent increases in SP protein content and PPT-mRNA levels in the cell bodies of the trigeminal ganglia. Previous studies have established that neurons in the trigeminal ganglia provide sensory innervation to the nasal cavity (Grunditz et al., 1994). Therefore, the data suggest that SP synthesis was increased due to nasal mucosal irritation resulting from the silica dust. Previous studies demonstrate chemical irritation by cigarette smoke, formalin, and sulfur dioxide can activate capsaicin-sensitive nerves, inducing nasal inflammation by the release of the mediator SP (Lundberg & Saria, 1983; Lundberg et al., 1987). An additional study demonstrated that PPT-mRNA production is increased in trigeminal ganglia cell bodies in response to toluene diisocyanate (TDI) exposure (Kalubi et al., 1992). However, the same study also demonstrated a decrease in SP immunoreactivity after TDI exposure. These differing results may be explained by the exposure time and technique used to irritate the nasal cavity. In the study by Kalubi and co-workers (1992), TDI was applied directly to the nasal cavity with a cotton bud for a few seconds. In the present study, silica dust was inhaled by the animals 8 h daily for 6 mo. The direct and brief nasal application of TDI to the nasal mucosa may induce immediate SP release from nasal mucosal nerve endings and consequently cause a lower SP protein content in the neuron cell body. In addition, as a nasal irritant, silica dust may also cause a direct release of SP from nerve endings in the nasal mucosa, but the extended exposure time may have allowed activation of higher PPT-mRNA expression in the cell body, increased translation, and thus higher SP protein levels. This neuronal response would allow increased neuropeptide levels for axonal transport from the cell bodies to the nerve endings for the release of SP.

Another explanation for alterations in neuropeptide levels may be that irritation by silica dust inhibits neutral endopeptidase (NEP) in the nasal epithelium. NEP is an enzyme that degrades tachykinins, like SP (Matsas et al., 1984; Skidgel et al., 1984), and modulates tachykinin activity in the airways (Borson et al., 1987). Previous studies demonstrated that cigarette smoke and TDI exposure decrease NEP activity, causing a lower level of SP degradation (Sheppard, 1988; Dusser et al., 1989), which enables a higher responsiveness to SP. The effect of silica on NEP has not been determined.

The increased SP synthesis in trigeminal ganglia neurons suggests that silica dust can activate sensory pathways that may be involved in nasal inflammation such as mucous edema and secretion. Earlier studies established that nasal irritation causes SP release from nerve endings in the nasal cavity, producing mucosa edema (Lundberg et al., 1983a, 1983b), and that the airway irritant acrolein causes depletion of SP from nerve fibers in the airway walls (Springall et al., 1990). More recent studies demonstrated increased SP levels and induction of neurogenic inflammation in the nasal mucosa after nasal allergen provocation with pollen and that pretreatment with a SP antagonist reduced the neurogenic inflam-

matory response (Nieber et al., 1991). Allergen challenge of sensitized animals also produces increased PPT message and content in nodose ganglion (Fischer et al., 1996). Our study suggests that nasal irritation by silica produces increased PPT mRNA levels, increased SP synthesis, and augmentation of SP content in neurons innervating the nasal mucosa. Thus, elevated SP production by neurons may contribute to exaggerated and prolonged inflammation and hyperresponsiveness in the nasal cavity. Although we have focused on the projections of trigeminal neurons to the nasal cavity, it should be noted that many of these neurons project to other structures in the head including the conjunctiva of the eyes, the skin of the face, and the oral mucosa, all of which may be irritated by silica exposure. Thus, some of the neuronal responses observed may have been initiated by irritation to the skin, eyes, or oral cavity.

The inhalation of silica dust has been associated mainly with classical pneumoconiosis and obstructive airway disease. However, our results suggest that silica dust can irritate the nasal mucosa and activate sensory nerve pathways in the upper airways. The alteration of SP neuronal responses in the trigeminal ganglia may be a possible explanation for upper airway neurogenic responses after the inhalation of silica dust and provide a possible beneficial mechanism designed to protect the distal airways.

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